

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

ZAUDERER *et al.*

Appl. No. 09/987,456

Filed: November 14, 2001

For: ***In Vitro* Methods of Producing
and Identifying
Immunoglobulin Molecules in
Eukaryotic Cells**

Confirmation No.: 6770

Art Unit: 1639

Examiner: Epperson, J.D.

Atty. Docket: 1821.0070004/EJH/T-M

Declaration Under 37 C.F.R. § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, Dr. Walter J. Storkus, residing at 3303 Mount Royal Boulevard,
Glenshaw, PA 15116, USA, declare and state as follows:

1. I was a member of the Scientific Advisory Board ("SAB") of Vaccinex, Inc.,
from 2001 until 2004.

2. A current *curriculum vitae* is appended hereto as Exhibit A1.

3. I received my Ph.D. degree in Microbiology and Immunology from Duke
University in 1986, evaluating the importance of MHC class I molecule expression by tumor
cells in their ability to be recognized and killed by natural killer (NK) cells. Since that time,
I have been an NIH-funded investigator targeting the development of tumor vaccines and
immunotherapies for patients with cancer. I am currently a tenured full professor in the

Departments of Dermatology and Immunology at the University of Pittsburgh. As seen from my attached *curriculum vitae*, I have published extensively, received many honors, and am involved in numerous professional and scientific societies related to immunology. Based on my education and experience, I am an expert in the field of immunology.

4. I have reviewed the above-identified patent application ("patent application"), the final Office Action dated April 21, 2005; PCT Publication No. WO 93/01296 to Rowlands *et al.* ("Rowlands"); PCT Publication No. WO 93/01296 to Zauderer ("Zauderer"); and Waterhouse *et al.*, *Nucleic Acids Res.* 21:2265-2266 (1993) ("Waterhouse") (collectively, "the cited references"). I have also reviewed the pending claims of the patent application.

5. The invention claimed in the patent application relates to the field of immunology. More particularly, the invention relates to the art or field of methods of identifying, producing, and/or expressing immunoglobulins in eukaryotic cells.

6. When Dr. Maurice Zauderer, a co-inventor of the captioned application and President and CEO of Vaccinex, Inc. ("Vaccinex") first presented the idea of the present invention to the SAB of Vaccinex, Inc., the SAB members and I were skeptical that the present invention would succeed. The reasons for my skepticism are explained in detail below.

7. The natural human immune system is necessarily an incredibly complex one that requires vast numbers of B cells interacting with antigen presenting cells in highly organized tissues in order to achieve the diversity necessary to produce antigen specific antibodies. At the time the idea for the present invention was presented to me, I did not think that antigen-specific antibodies could be efficiently selected from random libraries of immunoglobulin heavy and light chains expressed in eukaryotic cells *in vitro* because I thought specific antibodies of interest would occur at relatively low frequency and it would not be practical to screen the number of eukaryotic cells necessary in order to find an antibody that had specificity for a specific antigen of interest. I and the other members of the SAB were aware of reports that this could be done for libraries of antibody fragments expressed in phage. This did not, however, convince us that good antibodies could be selected in eukaryotic cells because: 1) the throughput for screening phage exceeded the expected throughput for screening libraries expressed in eukaryotic cells by as much as four orders of magnitude; 2) most of the work reported with antibody fragments expressed in phage was carried out with single chain Fv (scFv) in which the variable regions of immunoglobulin heavy and light chains are covalently linked, thereby increasing the likelihood that they will associate. This is significant because one of the concerns raised in the SAB was that antibodies are efficiently assembled and expressed in mature B lymphocytes because their component immunoglobulin heavy and light chains have been selected to pair properly. In contrast, we thought that random pairs of immunoglobulin heavy and light chains derived from separate libraries would be poorly matched and would, therefore, fail to associate properly in the eukaryotic cytoplasm. A related point is that antibody fragments expressed in phage, whether or not the immunoglobulin heavy and light

chain variable regions are covalently linked, concentrate and are assembled in the periplasmic space. The conditions of assembly in the eukaryotic cytoplasm are far different from those that apply in the periplasmic space and it could not be known what effect this would have on antibody assembly. This difference in the conditions of assembly may explain some of the many examples of antibodies selected from phage libraries that cannot be expressed in eukaryotic cells. Finally, 3) based on a biopharmaceutical industry survey commissioned by Vaccinex and carried out by L.E.K. Consulting (28 State St., Boston, MA 02109) in the Fall of 2001, "*Monoclonal Antibody Partnerships in the Biopharmaceutical Industry*" (attached hereto as Exhibit A2), we understood that the quality of antibodies selected from phage libraries was, at that time, viewed by senior industry executives as a research tool that, in comparison to immunoglobulin transgenic mice or humanization of murine antibodies, was not good enough or consistent enough for commercial therapeutic applications. Although Vaccinex management cited this as evidence of an unmet need for a library-based technology, it also highlighted the difficulty of the challenge. There were, at the time the present application was filed, no grounds for confidence that antibodies selected from libraries expressed in eukaryotic cells would be any more suitable for commercial therapeutic use than those selected from libraries expressed in phage.

8. Since the time the Vaccinex patent application was filed, there have been significant improvements to phage display technology and, especially, to the quality and diversity of immunoglobulin variable gene libraries expressed in phage. This has had a beneficial impact on the quality of antibodies selected using phage technology. Similarly, in spite of our concerns, selection of high quality antibodies from immunoglobulin gene libraries expressed in eukaryotic cells, as carried out at Vaccinex after the present

application was filed, has also proven quite successful. Apparently, random pairs of immunoglobulin heavy and light chains often do associate successfully and the efficiency of antibody assembly in eukaryotic cells is so much higher than in bacteria that antigen-specific antibodies can still be selected even though the throughput for screening libraries expressed in eukaryotic cells is much lower than for libraries expressed in phage. The key point is that, at the time the present application was filed, we could not have known that this technology would be successful.

9. The success of this invention would not have been expected based on the Rowlands, Zauderer and Waterhouse references. My expectations would not have changed in view of Rowlands, because Rowlands only demonstrated expression of a single antibody which had already been selected for immunoglobulin heavy and light chains that paired correctly and efficiently. This is a far simpler problem than expression of a large number of random pairs from two separate libraries. My expectations would also not have changed in view of Zauderer, because only one library was introduced into host cells. Therefore, Zauderer did not address the concern of assembling separate chains derived from two random libraries as in the present invention. Furthermore, although Waterhouse does suggest introducing a library of heavy chain fragments and a library of light chains into *E. coli* host cells for phage display of antibody fragments, I would not have expected that two random libraries could be introduced into eukaryotic host cells to provide antigen-specific antibodies. First, and most importantly, the Waterhouse technique was developed as an improvement for phage display, which is a system using prokaryotic host cells. As such, the Waterhouse technique could not simply be extrapolated into a eukaryotic host cell system. Second, in the specific examples of Waterhouse, it was not shown that using two libraries to

select a previously unidentified antigen-specific antibody would actually work in a prokaryotic phage display because the data described therein only shows the results of using a few immunoglobulin sequences that had previously been identified. I am aware of separate work by others (*e.g.*, H.J. de Haard *et al.*, *J Biol Chem*, 274:18218-18230, 1999, attached hereto as Exhibit A3) in which a library of heavy chain fragments and a library of light chains were introduced into *E. coli* host cells for phage display of antibody fragments, but, again, it would not have been possible to extrapolate from the conditions of assembly of antibody fragments in the periplasm of bacteria to assembly of immunoglobulin molecules in a eukaryotic system.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present patent application or any patent issued thereon.

Respectfully submitted,



Walter J. Storkus, Ph.D.

Date: 7/20/05

July 1, 2005

CURRICULUM VITAE

NAME: Walter J. Storkus, Ph.D.

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EDUCATION AND TRAINING

UNDERGRADUATE:

1977-1981	Brandeis University Waltham, MA 02154	B.A.	Biochemistry Mathematics
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GRADUATE:

1982-1986	Duke University Durham, NC 27710	Ph.D.	Microbiology/ Immunology
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Thesis: "NK Regulation of B cell Development"
Advisor: J.R. Dawson, Ph.D.

POST-GRADUATE:

1986-1987	Department of Microbiology and Immunology, Duke University Med. Ctr. Durham, NC 27710.	NIH Postdoctoral Fellow (J.R. Dawson, Ph.D.)
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1987-1991	Department of Microbiology and Immunology, Duke University Med. Ctr. Durham, NC 27710.	Research Associate (P. Cresswell, Ph.D./ J.R. Dawson, Ph.D.)
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APPOINTMENTS AND POSITIONS

ACADEMIC:

1985-1991	Department of Microbiology and Immunology, Duke University, Durham, NC 27710	Teaching Assistant
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HONORS

1999-2003	Member NIH Experimental Immunology Study Section
1995-1999	Received Cancer Research Institute Clinical Investigator Award
1996	Honored as "Outstanding Faculty" at Univ. Pittsburgh 1996 Honors Convocation, February 3, 1996.
1996	Klaus-Irmscher Lecturer, Wistar Institute, Philadelphia, PA, April 18, 1996
1997	Honored as "The 1997 Cancer Foundation of Western Australia visiting Professor of Cancer Research".

PUBLICATIONS-Refereed Articles

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3. Storkus, W.J. and Dawson, J.R.: B Cell sensitivity to natural killing: correlation with target cell stage of differentiation and state of activation. *J. Immunol.* 136:1542-1547, 1986.
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10. Storkus, W.J., Salter, R.D., Cresswell, P. and Dawson, J.R. Peptide-induced modulation of target cell sensitivity to natural killing. *J. Immunol.* 149: 1185-1190, 1992.
11. Storkus W. J., Zeh III, H. J., Salter R. D., Lotze, M. T. Identification of T cell epitopes: Rapid isolation of class I-presented peptides from viable cells by mild acid elution. *J. Immunother.* 14: 94-103, 1993.
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13. Storkus W. J., Zeh III, H. J., Salter R. D., Lotze, M. T. Isolation of human melanoma peptides recognized by class I restricted tumor infiltrating T lymphocytes. *J. Immunol.* 151: 3719-3727, 1993.
14. Zeh, H.J., Hurd, S., Storkus, W.J., and Lotze, M.T. Interleukin 12 promotes the proliferation and cytolytic maturation of immune effectors: Implications for the immunotherapy of cancer. *J. Immunother.* 14: 155-161, 1993.
15. Zeh, H.J. III, Salter, R.D., Lotze, M.T., and Storkus, W.J.. Flow cytometric determination of peptide-class I complex formation. *Human Immunol.* 39: 79-86, 1994.
16. Tahara, H.T., Zeh H.J. III, Storkus, W.J., Pappo, I., Watkins, S.C., Gubler, U, Wolf, S.F., Robbins, P.D., and Lotze, M.T. Fibroblasts genetically engineered to secrete interleukin-12 can suppress tumor growth and induce anti-tumor immunity to a murine melanoma *in vivo*. *Cancer Res.* 54: 182-189, 1994.
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 24. Tatsumi, T., Wesa, A., Finke, J.H., Bukowski, R.M. and **Storkus, W.J.** CD4+ T Cell-Mediated Immunity to Cancer. In: Cancer Immunotherapy at the Crossroads: How Tumors Evade Immunity and What Can Be Done? Bukowski, R. and Finke, J. (Eds.), The Humana Press, Totowa, NJ, pp. 67-86, 2004.
 25. Kalinski, P., Giermasz, A., Nakamura, Y., Basse, P., **Storkus, W.J.**, Kirkwood, J.M. and Mailliard, R.B. Helper role of NK cells during the induction of anticancer responses by dendritic cells. *Molecular Immunol.* 42: 535-539, 2005.

CLINICAL PROTOCOLS

- UPCI 94-21** "IL-12 Gene Therapy Using Direct Injection of Tumors with Genetically Engineered Autologous Fibroblasts", Principal Investigators: Hideaki Tahara, M.D., Ph.D. and Michael T. Lotze, M.D., **Co-Principal Investigator: Walter J. Storkus, Ph.D.**
- UPCI 94-95** "Randomized Phase I Evaluation of Immunization against Melan-A/MART-1, gp100, and Tyrosinase Peptides in Patients with Metastatic Melanoma Using MF59 Adjuvant", Clinical Principal Investigators, Michael T. Lotze, M.D. and John M. Kirkwood, M.D., **Laboratory Principal Investigator, Walter J. Storkus, Ph.D.**
- UPCI 95-043** "Randomized Phase I Evaluation of Immunization against Melan-A/MART-1, gp100, and Tyrosinase Peptides in Patients with Metastatic Melanoma Using rhIL-12 as Adjuvant", Clinical Principal Investigators, Michael T. Lotze, M.D. and John M. Kirkwood, M.D., **Laboratory Principal Investigator, Walter J. Storkus, Ph.D.**
- UPCI 95-060** "Randomized Phase I Evaluation of Immunization against Melan-A/MART-1, gp100, and Tyrosinase Peptides in Patients with Metastatic Melanoma Using Autologous Dendritic Cells Cultured with IL-4 and GM-CSF", Clinical Principal Investigators, Michael T. Lotze, M.D. and John M. Kirkwood, M.D., **Laboratory Principal Investigator, Walter J. Storkus, Ph.D.**
- UPCI 99-088** "Phase I-II Study of Vaccination with Melan-A/MART-1₅₁₋₇₃ and the Multi-epitope Vaccine containing Melan-A/MART-1₅₁₋₇₃ and the Melan-A/MART-1₂₇₋₃₅ Peptides in Patients with Metastatic Measurable Melanoma", Clinical Principal Investigators, John M. Kirkwood, M.D. and Hassane Zarour, M.D., **Co-Investigator: Walter J. Storkus, Ph.D. [Active]**
- UPCI 01-171** "Randomized Phase II Evaluation of Immunization Against Tumor Cells in Patients With Metastatic Melanoma Using Autologous Mature Dendritic Cells". Clinical Principal Investigators, John M. Kirkwood, M.D., **Co-Investigator: Walter J. Storkus, Ph.D. [Active]**
- ECOG E1696** "Phase II Evaluation of Immunization Against HLA-A2 Multiepitope Vaccine Containing Melan-A/MART-1, gp100 and Tyrosinase Peptides in Patients with Metastatic Melanoma", Study Chair: John M. Kirkwood, M.D., Study Co-Chair: **Walter J. Storkus, Ph.D. [Active]**

RESEARCH

1. GRANTS RECEIVED: CURRENT SUPPORT

"Dendritic cell-based therapies designed for murine tumors"

Principal Investigator: Walter J. Storkus, Ph.D.

Agency: National Institutes of Health

12% Effort

Type: 2R01 (CA 63350, Years 5-9) Period: April 1, 1997-March 31, 2006.

Evaluation of bone marrow-derived dendritic cell-based vaccines in murine tumor models.

"Vaccine Development for Oral Carcinoma"

Co-Investigator (Project 1, Core B): Walter J. Storkus, Ph.D.

12.5% Effort

Principal Investigator (Project 3): Walter J. Storkus, Ph.D.

Program Leader: Theresa Whiteside, Ph.D.

Agency: National Institutes of Health

Type: 1P01 (DE 12321, Years 5-9) Period: February 1, 1998-January 31, 2007.

Construction and assessment of DC-based vaccines for the treatment of squamous cell carcinoma of the head-and-neck (SCC-HN). Identification of MHC presented peptide epitopes derived from p53 protein that may serve as vaccine components and targets for immune monitoring of SCC-HN patients undergoing immunotherapy.

"Dendritic Cell Strategies to Elicit Tumor Reactive T Cells"

Principal Investigator: Walter J. Storkus, Ph.D.

10% Effort

Agency: National Institutes of Health

Type: 2R01 (CA 57840, Years 10-14) Period: May 1, 1994-April 30, 2008

Identification of multilineage tumor peptide epitopes recognized by CD4+ and CD8+ human T cells. Assessment of the immunogenicity of single or combined MHC class I- and II-presented peptide-based vaccines using dendritic cells *in vitro*. Design of clinical trials implementing defined MHC class I- and II-presented peptides and dendritic cells for the treatment of renal cell carcinoma.

"Dendritic Cell Biology and Therapy"

Co-Principal Investigator (Project 3): Walter J. Storkus, Ph.D. (with Louis D. Falo, Jr. M.D., Ph.D.) 10% Effort

Program Leaders: Olja Finn, Ph.D. and Louis D. Falo, M.D., Ph.D.

Agency: National Institutes of Health

Type: 2P01 (CA 73743, Years 6-10), Period: July 1, 1999-June 30, 2009.

Evaluation of the ability of DCs loaded with various tumor antigen formats (lysate, co-cultures, fusions) to elicit anti-tumor T cells in murine tumor models and in a phase I clinical trial for the treatment of melanoma. Evaluation of the ability of DCs to repolarize Th2-type T cell immunity to Th1-type immunity *in vitro* in patients with melanoma.

"Polarization of Dendritic Cells by CD8+ T cells"

Co-Investigator: Walter J. Storkus, Ph.D.

10% Effort

Principal Investigator: Pawel Kalinski, M.D., Ph.D.

Agency: National Institutes of Health

Type: 1R01 (CA 95128, Years 1-5), Period: July 1, 2003-June 30, 2007

This project tests the hypothesis that primed Type-1 CD8+ T cells condition DCs to become increasingly DC1-like, and in turn, these APCs may become better stimulators of Th1-type CD4+ T cell responses *in vitro*. Based on pre-clinical data derived from this project, a phase I/II clinical trial is proposed to assess this hypothesis in melanoma patients. No overlap with current submission.

"Impaired Tumoricidal Activity of DCs in HNC Patients"

Co-Investigator: Walter J. Storkus, Ph.D.

5% Effort

Principal Investigator: Nikola Vujanovic, Ph.D.

Agency: National Institutes of Health

Type: 1R01 (DE14775, Years 1-5), Period: July 1, 2002-June 30, 2007

This project has the goal to investigate the mechanisms of decreased expression of TNF family ligands by and impaired tumoricidal activity of dendritic cells (DCs) in head and neck cancer (HNC) patients, as well as the immunological consequences and reparation of these defects by simultaneous transfer of normal TNF, FasL and TRAIL genes into DCs. No overlap with current submission.

"Cytokine Gene Therapy for Cancer-Preclinical Studies"

Program Leader, Project 2 PI, Core A (Administrative) PI: Walter J. Storkus, Ph.D.

20% Effort

Agency: National Institutes of Health

Type: 1P01 (CA 100327; Years 1-5), Period: December 1, 2004-November 30, 2009.

Analysis of DC-based cytokine gene therapy of gliomas and spontaneous metastatic melanoma models in mice. Specific cytokines evaluated include: Type I IFNs (Project 1), IL-12 (Project 2) and IL-18, IL-1H4, IL-23 and IL-27 (Project 3).

"Combinational Immunotherapies Targeting Tumor RTKs"

Principle Investigator: Walter J. Storkus, Ph.D.

15% Effort

Agency: National Institutes of Health

Type: 1P01 (CA 114071-01; Years 1-5), Period: April 1, 2005-March 30, 2010.

Evaluation of RTK overexpression and the ability of ligand agonists and PTP inhibitors to promote their proteasomal degradation and enhanced recognition by T cells in the setting of renal cell carcinoma (RCC). Performance of a phase I clinical trial incorporating DC/RTK peptide-based vaccination and anti-RTK Ab therapy in patients with accessible metastatic RCC.

2. GRANTS RECEIVED: PREVIOUS SUPPORT AS PI

“Immunobiology of Atypical Nevi”

Principal Investigator: Walter J. Storkus, Ph.D.

Agency: National Institutes of Health

Type: 1R01 (CA 82297, Years 1-5) Period: July 1 1999-April 30, 2004.

Analysis of immune function and specificity in pre-malignant nevi. Evaluation of T cell and dendritic cell infiltration and functional status as correlated with the degree of atypia in situ. Analysis of the expression of melanoma-associated antigens during the course of disease progression and the in situ immune response in melanoma patients at high-risk for recurrence.

“Phase I Clinical Trials of Melanoma Peptide-Based Vaccines”

Principal Investigator: Walter J. Storkus, Ph.D.

Agency: Cancer Research Institute

Type: Period July 1, 1995-June 30, 1999.

Evaluation of peptide-pulsed dendritic cells (DC) as vaccines to promote anti-melanoma reactive CD8+ T cells.

3. PATENTS (AWARDED/Submitted)

“Elution And Identification of T Cell Epitopes From Viable Cells”, Inventors: **Walter J. Storkus, Ph.D.** and Michael T. Lotze, M.D., A method to isolate and identify T cell epitopes., U.S. Patent Number 5,989,565 (Issued 11/23/99).

“Methods for isolation and use of T cell epitopes eluted from viable cells in vaccines for treating cancer patients”, Inventors: **Walter J. Storkus, Ph.D.** and Michael T. Lotze, M.D., A method to construct cancer vaccines based on T cell epitopes eluted from viable tumor cells, U.S. Patent Number 6,077,519 (Issued 6/20/00).

“EphA2 Peptide Epitopes and Uses Therefor”, Inventor: **Walter J. Storkus, Ph.D.** and Michael S. Kinch, Ph.D. Use of molecularly-defined EphA2 peptides for the treatment of patients with cancer. Patent Pending.

“Identification of an IL-13 Receptor Alpha2 Peptide Analogue Capable of Enhancing Stimulation of Glioma-Specific CTL Responses”. Co-Inventor: **Walter J. Storkus, Ph.D.** Provisional Patent Submitted.

4. SEMINARS AND INVITED LECTURESHIPS RELATED TO RESEARCH: (RECENT INVITED TALKS, GIVEN 1999-2004)

Perspectives in Melanoma III, New Orleans, LA, March 18, 1999. Title: “**Induction of cellular immune responses using adjunctive therapy for the prevention of melanoma relapse**”.

Oncology Grand Rounds, New York University, New York City, NY, April 5, 1999. Title: “**Cancer Immunotherapy: Preclinical and clinical studies of dendritic cell-based vaccines**”.

International Workshop “Immunology of Ocular Tumors”, Ettal, Germany, April 8-9, 1999. Title: “**Dendritic APC pulsed with peptide/protein tumor antigens as vaccines**”.

First International Kidney Cancer Symposium, Chicago, IL, October 1-3, 1999. Title “**Dendritic Cells in RCC**”.

14th International Convocation on Immunology. Cancer Immunotherapy: Pitfalls/Solutions. Amherst, NY, October 8-11, 1999. Title: “**Tumor Antigens and Dendritic Cell-Based Therapy**”.

Second Course on Immunotherapy and Gene Therapy of Cancer: Experimental basis and Clinical Applications, Hospital Clinico Universitario, Zaragoza, Spain. November 9-12, 1999. Title: **"Dendritic Cells and their Clinical Application to Cancer Vaccines"**.

IX Congresso Nazionale, Societa Italiana di Urologia Oncologia. "Tumori Del Rene E Surrene". Sheraton Nicolaus Hotel, Bari, Italy, November 28-30, 1999. Title: **"Immunotherapy of Renal Cell Carcinoma: Basic Concepts"**.

2nd Annual Walker's Cay Colloquium on Cancer Vaccines and Immunotherapy, Walker's Cay Resort, Abaco, Bahamas, March 8-11, 2000. Title: **"Epitope Spreading and Objective Clinical Responsiveness to Tumor Peptide-Based Vaccines"**.

Melanoma Consensus Conference. Four Seasons Hotel, Atlanta, GA, April 7-9, 2000. Title: **"Future Directions: Basic Science Issues"**.

Perspectives in Melanoma IV. State of the Art at the Turn of the Century. The Pittsburgh Hilton, Pittsburgh, PA, June 1-2, 2000. Title: **"Melanoma Antigens Recognized by CD8+ T cells"**.

Integrated Therapeutics Group "Advances in Melanoma Management" Investigator's Meeting, Sheraton Chicago Hotel and Towers, Chicago, IL, October 19-22, 2000. Title: **"Overview of peptide vaccine trials"**.

The Chicago Association of Immunologists, Amour Academic Facility at Rush-Presbyterian St. Luke's Medical Center, Chicago, IL, November 7, 2000. Title: **"Dendritic Cell Subsets and Immunotherapy"**.

Immunology Seminar Series, Roswell Park Cancer Institute, Buffalo, NY, November 14, 2000. Title: **"DC Subsets and Epitope Spreading: Critical Issues in Cancer Vaccines"**.

Combined Immunology/Cancer Center Seminar Series, Indiana University, Indianapolis, IN, December 20, 2000. Title: **"DC Precursor Mobilization and In Situ Maturation as an Approach for Cancer Immunotherapy"**.

Biochemistry and Molecular Biology Seminar Series, Purdue University, W. Lafayette, IN, January 22, 2001. Title: **"DC subsets, Epitope Spreading and Clinical Responsiveness to Cancer Vaccines"**.

5th World Conference on Melanoma, San Georgio Island, Venice, ITALY, February 28-March 3, 2001. Title: **"Epitope Spreading in Melanoma Patients"**.

3rd Annual Walker's Cay Colloquium on Cancer Vaccines and Immunotherapy, Walker's Cay Resort, Abaco, Bahamas, March 7-10, 2001. Title: **"Epitope Spreading in Cancer Vaccines"**.

Immune Monitoring Workshop, Society of Biological Therapy, Co-Chair and Discussant in "Tetramer Analyses" Working Group Session, Natcher Center, NIH, Bethesda, MD, November 8, 2001.

Annual Meeting of the Society of Biologic Therapy, Co-Chair and Speaker in "Vaccine/Dendritic Cells" Concurrent Session, Natcher Center, NIH, Bethesda, MD, November 9, 2001. Title: **"Th1/Th2 Responses in RCC and Melanoma"**.

5th Annual Cancer Center Consortium Meeting, Marriott Downtown, Cleveland, OH, February 21-23, 2002. Title: **"Autoimmunity, Epitope Spreading and Cancer Therapy"**.

4th Annual Walker's Cay Colloquium on Cancer Vaccines and Immunotherapy, Walker's Cay Resort, Abaco, Bahamas, March 6-9, 2002. Title: **"CD4+ T cell responses in melanoma/RCC and DC-based repolarization"**.

Immunology Council Meeting, The Johns Hopkins University, Baltimore, MD, March 18th, 2002. Title: **"Disease-Associated Bias in CD4+ T-helper Responses to Tumor Antigens in RCC and melanoma"**.

Simmons/Billiar Research Conference, Department of Surgery, University of Pittsburgh, Pittsburgh, PA, August 26th, 2002. Title: **"Disease-Stage Dependent Deviation in the Anti-Tumor Th Response"**.

CME Course on Innovations in Oncology, University of Pittsburgh, Pittsburgh, PA, September 13th, 2002. Title **"Melanoma Vaccines: Progress and Potential"**.

Oral Cancer Center Seminar Series, University of Pittsburgh, Pittsburgh, PA, November 29th, 2002. **"CD4+ T cell Dysfunction in Patients with Cancer"**.

Cleveland Clinic Immunology Seminar, Cleveland Clinic Foundation, Cleveland, OH, April 10th, 2003. **"CD4+ T Cell Immune Dysfunction in Patients with RCC and Corrective Immunotherapy"**.

The First Melanoma Research Conference, Wydham Franklin Plaza Hotel, Philadelphia, PA, June 21-24, 2003. **"Repolarizing Anti-Melanoma Th Responses"**.

Perspectives in Melanoma VI, Sheraton Bal Harbour Beach Resort, Miami, FL, November 13-14, 2003. **"Melanoma induced deviation in CD4+ T helper cell functional polarization"**.

Cleveland Clinic Oncology Grand Rounds, Cleveland Clinic Foundation, Cleveland, OH, January 28th, 2004. **"Combinational Immunotherapies Targeting RTKs"**.

1st International Congress on Kidney and Bladder Cancer, Ritz-Carlton Hotel, Orlando, FL, August 15th, 2004. **"T Cell Responses to Tumor Associated Antigens"**.

Interdisciplinary Graduate Program in Immunology Seminar Series, University of Iowa, Iowa City, IA, September 15th, 2004. **"RTKs as targets for immune intervention in cancer: An EphA2 Model"**.

Cytokines in Cancer and Immunity, Joint Meeting of International Cytokine society and International Society for Interferon and Cytokine Research, Hilton Caribe Hotel, San Juan, PR, October 21-25, 2004. Title: **"IL-12 Family Cytokine Gene Therapy of Cancer"**.

The Third International Kidney Cancer Symposium, Palmer House Hilton, Chicago, IL, November 12-14th, 2004. Title: **"RCC Antigens and the Immune Response"**.

Dana Farber/Harvard Cancer Center Renal Cancer Program and Renal Cancer SPORE Minisymposium, Jimmy Fund Auditorium, Boston, MA, March 31, 2005. Title: **"Combination immune targeting of receptor tyrosine kinases (RTKs) in renal cell carcinoma"**.

5. OTHER RESEARCH-RELATED ACTIVITIES:

1986-Present	Ad-Hoc Journal Review: <i>The Journal of Immunology</i> , <i>Nature</i> , <i>Human Immunology</i> , <i>The International Journal of Cancer</i> , and <i>The Journal of Immunotherapy</i> , <i>Cancer Research</i> , <i>Science</i> , <i>Blood</i> , <i>Immunity</i> , <i>Science</i> and <i>Nature Medicine</i> .
1991-Present	Member, Pittsburgh Cancer Institute/University of Pittsburgh Cancer Institute.
1994-Present	Member, Editorial Board for the journal <i>Natural Immunity</i> .
1995	Member, NCI Special Review Study Section (RFA CA 94-08), Title: National Cooperative Drug Discovery Groups, Bethesda, MD., January 24-26, 1995.
1994-1996	Ad-Hoc Reviewer for Grants submitted to the Dutch Cancer Society, the Israeli Science Foundation, and the National Science Foundation (U.S.).
1996	Member, NCI Special Review Study Section (RFA CA 95-17), Title: Cancer Therapy with Biologic Response Modifiers (CATBRM), Bethesda, MD, June 11-13, 1996.
1999-2003	Member, Experimental Immunology Study Section, NIH.

2000 NIH GCRC Site Visit, University of Chicago, April 11-12, 2000.

2000 NIH Program Project Site Visit, University of Pennsylvania, NCI-C GRB-2 (E3), June 24-26, 2000.

2000 Department of Defense (DOD) Prostate Cancer Research Program Grant Review, Vienna, VA, July 6-8, 2000.

2000 NIH Center for Scientific Review, Special Emphasis Panel, ZRG1 ET-1 (02), July 07, 2000.

2000 NIH Experimental Therapeutics-2; Ad Hoc Reviewer, October 23-25, 2000.

2001 External Advisor for the University of Chicago's NIH grant PO1 CA 74182 (Hans Schreiber, Program Leader), November 7, 2000.

2001-2003 Scientific Advisory Board Member, Vaccinex, Inc. (Rochester, NY).

2002 External Advisor to Cervical Cancer SPORE, University of Alabama-Birmingham (Dr. Ronald D. Alvarez, SPORE Director).

2002-Present Co-Program Organizer for "Tumor Immunology and Immunotherapy" Block for AAI annual meeting 2002.

2002-Present Section Editor, *The Journal of Immunology*

2002 NCI-CCSG Site Visit Member, Wistar Institute (Kaufman), February 24-26, 2003.

2003 NIH Ad-Hoc Reviewer for ZRG1 SSS-F (02) SEP. August 5, 2003.

2003 NIH Ad-Hoc Reviewer for ZAI1 PTM-1 (J3) "Innovative Grants on Immune Tolerance". November 10-12, 2003.

2003 Ad-Hoc Reviewer for Grant Applications submitted to the Melanoma Research Foundation, November 20, 2003.

2003 NIH Ad-Hoc Reviewer for ZRG1 DT-02 SEP. November 24, 2003.

2003 Editor (Basic Science), *Melanoma Research*

2004 NIH Program Project Site Visit, University of Pennsylvania, NCI-C GRB-1 (MC), P01 CA 109095-01, El-Deiry, February 23-25, 2004.

2004-2006 Member, NCI's Biological Resource Branch Oversight Committee (BRB/OC).

2004-present External Advisor to Breast Cancer SPORE, Fox Chase Cancer Center, (Dr. Jose Russo, SPORE Director).

2004 NCI-CCSG Site Visit Member, University of North Carolina, Chapel Hill, NC, May 24-26, 2004.

2004 Ad Hoc Reviewer for Grant Applications submitted to the Melanoma Research Foundation, September 21, 2004.

2004 Ad-Hoc Reviewer for NIH TTT Immunology IRG, October 28-29, 2004.

2004-2006 Member, NIH Parent Sub-Committee C

2005 Ad-Hoc Reviewer for NCI P01 Experimental Therapeutics B, June 6-7, 2005.

SERVICE

1. UNIVERSITY AND MEDICAL SCHOOL

1986-1991 Post-Doctoral Representative to Divisional Post-Doctoral Admissions Committee, Dept. Microbiology and Immunology, Duke University, Durham, NC.

1992-1993 Ad-Hoc Reviewer. Institutional American Cancer Society Awards (PCI).

1993-1994 Ad-Hoc Reviewer. Competitive Medical Research Fund (Presbyterian Hospital).

1993-2000 Member, University of Pittsburgh ACS Institutional Grant Review Board.

1996-2000 Member, Program in Immunology, Immunology Curriculum Committee.

1996-2000 Member, UPCI Health Sciences Research Committee.

2000 Organized UPCI Biologic Therapy Retreat, June 19, University of Pittsburgh Johnstown Campus, The Living/Learning Conference Center, Johnstown, PA.

2001-2003 Member, Standing Committee for Tenured Faculty Promotions and Appointments, School of Medicine, University of Pittsburgh, Pittsburgh, PA.

2002-present Member, Molecular Medicine Institute of the University of Pittsburgh, Pittsburgh, PA.

2003-present Co-Director, Cancer Program of the Molecular Medicine Institute of the University of Pittsburgh, Pittsburgh, PA.

2. TEACHING

1986-1990	Teaching assistant for First Year Graduate Student Immunology Course, Fall Semester, Dept. of Microbiology and Immunology, Duke University.
Fall 1992	Comprehensive Immunology (MSMIC 2360). Dr. C. Milcarek organizer. 2 Lectures, "NK cells" and "Mechanisms of Cytolysis".
Spring 1993	First Year Medical School Immunology Block, <u>Infection and Inflammation</u> . Dr. B. Rabin organizer. 1 Lecture, "T lymphocytes". Course facilitator for problem based learning cases.
Spring 1993, 1994.	Immunology and Human Disease (MSMIC 2220). Dr. O. Finn, organizer. 1 Lecture, "Tumor Antigens".
Fall 1993-1996	Cell structure and Function (MSNAC 2152). Dr. C. C. Widnell organizer. 2 Lectures, "Non-adaptive Immunity" and "T-cell Differentiation".
Spring 1994	First Year Medical School Immunology Block, <u>Infection and Inflammation</u> . Dr. B. Rabin organizer. 2 Lectures, "The Major Histocompatibility Complex" and "Antigen-Presenting Cells". Course facilitator for problem based learning cases.
Spring 1995	First Year Medical School Immunology Block, <u>Infection and Inflammation</u> . Dr. B. Rabin organizer. 1 Lecture, "Molecular Basis of T cell Antigen Recognition and Activation".
Fall 1995	Comprehensive Immunology (MSMIC 2360). Drs. C. Milcarek and S. McCarthy co-organizers. 4 Lectures, "Antigen Processing I/II" and "NK Cells I/II".
Spring 1996	First Year Medical School Immunology Block, <u>Infection and Inflammation</u> . Dr. B. Rabin organizer. 1 Lecture, "Molecular Basis of T cell Antigen Recognition and Activation". Course facilitator for problem based learning cases.
Spring 1997	First Year Medical School Immunology Block, <u>Infection and Inflammation</u> . Dr. B. Rabin organizer. 2 Lectures.
Fall 1999	Contemporary Topics in Immunology (MSIMM 3220). Co-Course Director (Lou Falo, M.D., Ph.D., Co-Director) Semester Topic: Dendritic Cells: Biology and Function, 15 sessions.
Spring 2000, 2001	Immunology of Human Disease (MSIMM 3230), Title "Tumor antigens and vaccines".
Spring 2002	First Year Medical School Immunology Block, <u>Infection and Inflammation</u> . Drs. R. Duquesnoy and M. Shurin organizers. 1 Lecture: "T-cell Receptor, MHC, Antigen Processing and Presentation", January 3, 2002.
Fall 2002	Immunology of Human Disease (MSIMM 3230), Title "Tumor antigens", November 8, 2002.
Spring 2003	First Year Medical School Immunology Block, <u>Infection and Inflammation</u> . Drs. R. Duquesnoy and M. Shurin organizers. 1 Lecture: "T-cell Receptor, MHC, Antigen Processing and Presentation", January 2, 2003.
Spring 2004	Immunology of Human Disease (MSIMM 3230), Title "Tumor antigens", March 26, 2004.

TEACHING (Committee Work/Research Supervision)

Pre-Doctoral:

1993-1995	Chairman, Ph.D. Thesis Committee for Mr. Matt Tector (Pathology), graduated with Ph.D. 5/95.
1994	Member, Comprehensive Exam Committee for Mr. Andrew Nowalk (MGB).
1995	Supervisor, Summer Laboratory Internship by U. Pittsburgh Medical School Student Mr. Thomas Zavoral.
1996	Member, Comprehensive Exam Committee for Dr. Ira Berman, M.D. (MGB), graduated 6/98.
1996-1997	Member, M.S. Thesis Committee for Mrs. Amy Mank-Seymour (GSPH), graduated 3/97.
1996-1997	Member, Ph.D. Thesis Committee for Mr. Mark Alter (MGB), graduated 6/97.
1996-1998	Member, Ph.D. Thesis Committee for Ms. Lian Zheng (GSPH), graduated 5/98.
1999-2002	Member, Ph.D. Thesis Committee for Ms. Melina Soares (MGB), graduated 8/02.
1999-2002	Member, Ph.D. Thesis Committee for Ms. Anda Vlad (MGB), graduated 10/02.
1998-2002	Member, Ph.D. Thesis Committee for Ms. Kendra Bodner (MVM), graduated 1/02.
1999-present	Member, Ph.D. Thesis Committee for Ms. Bonnie Colleton (GSPH).
1998	Chair, Comprehensive Exam Committee for Mr. Jayakar Nayak (IMM).
1997-2001	Member, Comprehensive Exam Committee and M.S. Thesis Committee for Ms. Cecilia

Vasquez, graduated 3/01.

2000-2002 Member, Comprehensive Exam Committee and Ph.D. Thesis Committee for Ms. Lori Stolinski /Spencer (IMM), graduated 2/02.

1999-present Ph.D. Supervisor of Mr. Lazar Vujanovic (IMM), expected 12/05.

2000-present Co-Supervisor of Ph.D. Candidate Ms. Anna Kalinska (University of Amsterdam).

2000 Chairman, Comprehensive Exam Committee for Ms. Jessica Kettel (IMM), graduated 8/03.

2000-present Ph.D. Supervisor of Mr. Aklile Berhanu (IMM), expected 7/05.

2000-2004 Ph.D. Supervisor of Mr. Dominic Warrino (IMM), graduated 3/04.

2000-2004 Ph.D. Supervisor of Mr. Christopher Herrem (IMM), graduated 6/04.

2000-present Member, Comprehensive Exam Committee and Ph.D. Thesis Committee for Ms. Bridget Colvin (IMM), graduated 12/04.

2000-2002 Member, Ph.D. Thesis Committee for Mr. Paul Hu (IMM), graduated 6/02.

2001 Chairman, Comprehensive Exam Committee for Mr. Nehad Alajez (IMM), graduated 10/03.

2001-present Member, Ph.D. Thesis Committee for Mr. David Hokey (IMM).

2002-2005 Member, Comprehensive Exam Committee and Ph.D. Thesis Committee for Mr. Timucin Taner (IMM), graduated 2/05.

2002-present Ph.D. Supervisor of Mrs. Jian Huang (IMM), expected 8/05.

2002 Member, Comprehensive Exam Committee for Ms. Pamela Beatty (IMM), Ms. Casey Carlos (IMM), Ms. Kavitha Rao (IMM).

2004-present Ph.D. Supervisor of Mrs. Mayumi Kawabe (IMM).

Post-Doctoral:

1992-1994 Co-supervisor, Post-Doctoral Research for Drs. Tohru Itoh (M.D., Dept. Surgery) and Markus Mauerer (M.D., Dept. Surgery). Currently Assistant Professor of Surgery, University of Osaka, JAPAN and Associate Professor of Dermatology, University of Mainz, GERMANY, respectively.

1992-1995 Co-supervisor, Post-Doctoral Research for Dr. Jose I. Mayordomo (M.D., Dept. Surgery) and Dr. Laurence Zitvogel (M.D., Dept. Surgery). Currently Associate Professor of Medical Oncology, University of Zaragoza, SPAIN and Associate Professor of Medicine, Institut Gustave Roussy, Villejuif, FRANCE, respectively.

1995-1998 Co-supervisor, Post-Doctoral Research for Dr. Bettina Couderc (Ph.D., Dept. Molecular Genetics and Biochemistry). Currently Assistant Professor of Pharmaceutical Sciences, University of Toulouse, FRANCE.

1996-1999 Supervisor, Post-Doctoral Research for Dr. Thomas Tueting (M.D., Dept. Surgery). Currently Associate Professor of Dermatology, University of Berlin, GERMANY.

1997-2000 Supervisor, Post-Doctoral Research for Dr. Lisa Salvucci Kierstead (Ph.D., Dept. Surgery). Currently Staff Scientist, Merck & Co., Wayne, PA, USA.

1998-1999 Supervisor, Post-Doctoral Research for Dr. Loreto Gesualdo (M.D., Dept. Surgery). Currently Associate Professor of Nephrology, University of Bari, ITALY.

1998-2000 Supervisor, Post-Doctoral Research for Dr. Wolfgang Herr (M.D., Dept. Surgery). Currently Assistant Professor of Surgery, University of Mainz, GERMANY.

1998-2001 Supervisor, Post-Doctoral Research for Dr. Elena Ranieri (Ph.D., Dept. Surgery). Currently Associate Professor of Nephrology, University of Foggia, ITALY.

1998-2002 Supervisor, Post-Doctoral Research for Dr. Jan Mueller-Berghaus (M.D., Dept. Surgery). Currently Assistant Professor of Laboratory Medicine, University of Mannheim, GERMANY.

1999-2001 Supervisor, Post-Doctoral Research for Dr. Pia Bjorck (Ph.D., Dept. Dermatology). Currently Assistant Professor of Medicine, Stanford University, Stanford, CA, USA.

2001-2004 Supervisor, Post-Doctoral Research for Dr. Eva Pizzoferrato (Ph.D., Dept. Surgery). Currently Instructor of Medicine, University of Pittsburgh.

2001-2003 Supervisor, Post-Doctoral Research for Dr. Tomohide Tatsumi (M.D., Ph.D., Dept. Surgery). Currently Assistant Professor of Molecular Therapeutics, Osaka University School of Medicine, JAPAN.

2002-2004	Supervisor, Post-Doctoral Research for Dr. Amy Wesa (Ph.D., Dept. Surgery). Currently Instructor, Department of Dermatology, University of Pittsburgh.
2005-present	Supervisor, Post-Doctoral Research for Dr. Hideo Komita (Ph.D., Dept. Dermatology).

LEK.

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***MAb Partnership Strategy
Final Presentation***

November 1, 2001

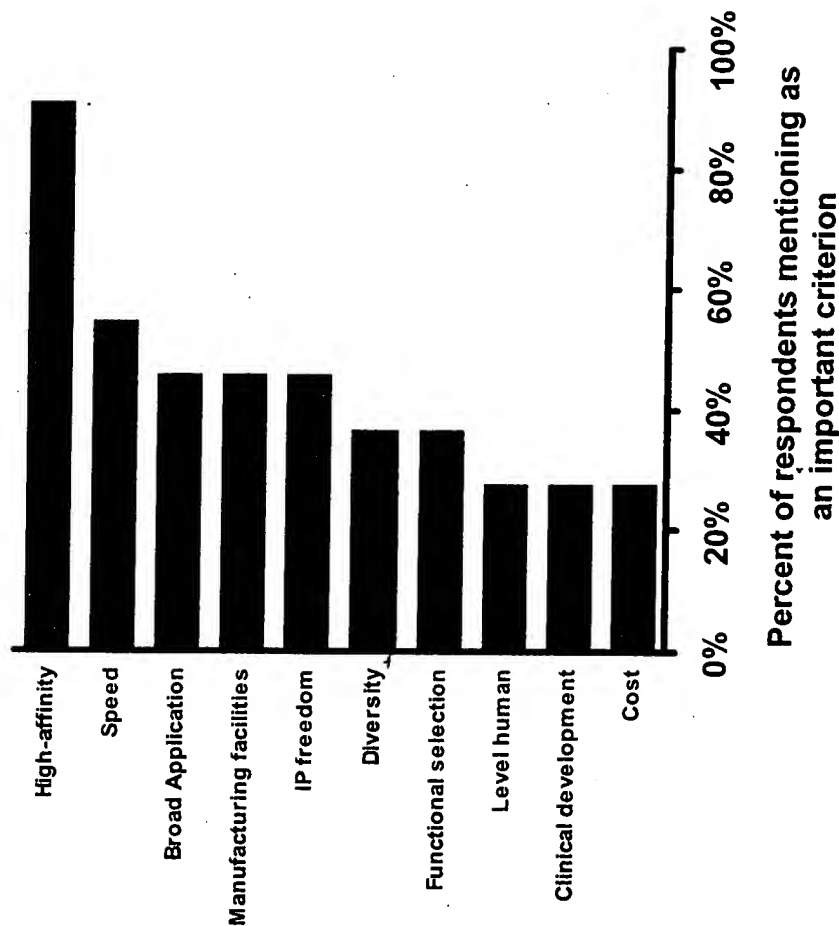
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The materials contained in this document are intended to supplement a discussion between Vaccinex and L.E.K. Consulting on November 1, 2001
These perspectives are confidential and will only be meaningful to those in attendance

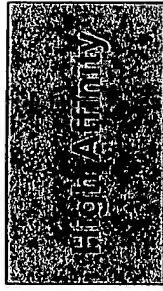
CUSTOMER ANALYSIS - DECISION CRITERIA

Interviewees listed the affinity of the end product as the key factor in determining which MAb discovery platform to use



- Interviewees were asked what the most important criteria were in selecting a MAb partner and the mentions of various criteria were scored
- Interviewees consistently mentioned wanting an antibody selection platform that reliably worked and would generate a high-affinity MAb
- While high cost could be a deal killer, it was mentioned by only three companies as one of their key criteria

Of primary importance is the delivery of a high affinity MAb, regardless of the other concerns



- There is still demand for a novel platform that delivers high affinity MAbs
“... I think it's quite obvious what the field is looking for, we want something that works!...”

David Wurtman, EOS Biotechnology, Director of Business Development

- Interviewees thought the biological process of affinity maturation which occurs in the in vivo mouse immune response significantly improves the quality of antibodies

“... We want a high affinity antibody from the start. The in vivo approach is better for at this and why we use XenoMouse first, and if that fails, we try normal mice... speed is nice but quality is the primary driver...”

Ian Nisbet, Millennium Pharmaceuticals, Director of Licensing and M & A

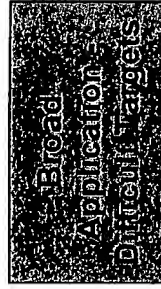
- This opinion is reflected in the high number of deals signed over the last 5 years by transgenic mouse companies (50% of deals)

- However, because the mouse approach doesn't consistently work for all targets and the phage approach commonly yields low affinity antibodies, no current platform satisfies everyone

“... We have been evaluating MAb companies for research and therapeutic uses for awhile now and we have yet to make a decision. None of the companies are a clear winner...”

Chi Chi Zhu, Celera Genomics, Director of Business Development

Interviewees also saw opportunities with our platform to screen difficult antigens and potentially screen based on functional assays



- Companies thought this approach would be useful for a variety of targets for which they had been unsuccessful in generating high affinity antibodies

"... 50% of our targets fail to elicit an immune response in mice..."

Ian Nisbet, Millennium Pharmaceuticals, Director of Licensing and M & A

"... There's no question this is where the mouse falls down. In oncology, the majority of our targets fail to work...this is absolutely due to the difficulties with homologous targets..."

Paul Spence, Pharmacia, Executive Director of Biotechnology

"... I would be interested in whether their approach would be useful for non-protein targets. We have several which have been particularly difficult to develop antibodies against..."

Susan Thorpe, Novo Nordisk, Program Manager Scientific Licensing

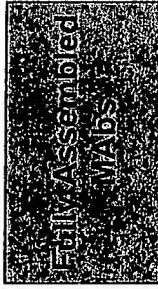
- Several were quite interested in selecting antibodies based upon functional assays

"... What is the application range on the functional assays. We are working with anti-fungals and if there was a way to screen for antibodies based upon fungal cell adherence or growth, we would be quite interested..."

Richard Labaudiniere, Genome Therapeutics, SVP of Research & Development

"... I see this platform fitting in where we understand the desired effector function and want to use that in the screen. Functional assays get you straight to the bottom line..."

Paul Spence, Pharmacia, Executive Director of Biotechnology

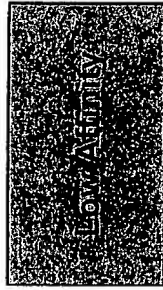


- Customers thought the generation of fully assembled human MABs would make our approach superior to other phage display platforms

"... Here you can have assays setup to immediately test the IgG isotype for the particular effects you want to see. Other phage approaches require re-engineering steps to clone the selected fragments into a full antibody structure..."

Paul Spence, Pharmacia, Executive Director of Biotechnology

...however, there were some concerns regarding the affinity levels of our MABs and the general lack of data



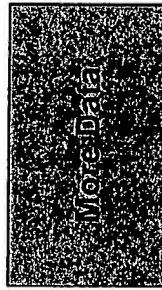
- There was skepticism regarding whether the library diversity would be sufficient to produce high-affinity MABs without affinity maturation

"... We consider phage display to be a research tool. We've never seriously considered it for therapeutic purposes due to the low quality of the antibodies produced. Leukosite had a deal with MorphoSys for 3 targets but that has ended..."

Ian Nisbet, Millennium Pharmaceuticals, Director of Licensing and M & A

"... We use mice and if that doesn't work, we use rabbits. The in vivo affinity maturation in those systems produces better antibodies than the phage systems..."

Dee Atwell, Celltech, Business Development Manager



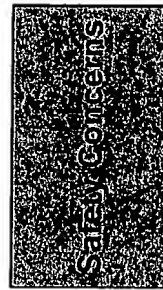
- The majority of interviewees wanted to view data demonstrating successes in MAB discoveries and positive comparisons versus other platforms

"... I would want to see some results and a presentation of why it is superior. It seems smart on paper, but I would want to see some examples..."

Richard Labaudiniere, Genome Therapeutics, SVP of Research & Development

"... Abgenix will be a yardstick for our future deals. Vaccinex will have to demonstrate how their approach is better than what we are currently using ..."

Andrew Pasternak, MDS Proteomics, Manager of Technology Development



- Several people mentioned safety concerns with handling vaccinia virus, but were typically content as long the experiments were to be handled by us

"... What is the biosafety surrounding vaccinia. Are there additional regulations. It must be harder to handle than yeast or *E. coli*..."

Ueli Gubler, Roche, Senior Research Leader

Our platform can be positioned as an alternative to mouse approaches for targets that failed to produce an antibody in mice

- Companies often de-prioritize targets that fail to generate MAbs, and thought partnerships involving such targets would be low risk with high potential rewards

“... One of our selection criteria in advancing a project is whether we can generate antibodies against the target. We may be interested in re-examining some of these deprioritized targets...”
Ian Nisbet, Millennium Pharmaceuticals, Director of Licensing and M & A

“... From target to target, it is sometimes difficult to generate an immune response in a mouse. This is definitely the selling point of phage...”
Dee Atwell, Celltech, Business Development Manager
- We can make favorable comparisons versus phage display approaches due to our human assembly in human cells, immediate generation of bivalent immunoglobulins, and improved diversity due to independent heavy and light chain selection
- With experience and further positive data on the quality of antibodies generated, we can then begin to position the platform as superior in terms of both breadth of targets and high quality

- Richard Labaudinière, Genome Therapeutics, SVP of Research & Development**

A Large Non-immunized Human Fab Fragment Phage Library That Permits Rapid Isolation and Kinetic Analysis of High Affinity Antibodies*

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We report the design, construction, and use of the first very large non-immunized phage antibody library in Fab format, which allows the rapid isolation and affinity analysis of antigen-specific human antibody fragments. Individually cloned heavy and light chain variable region libraries were combined in an efficient two-step cloning procedure, permitting the cloning of a total of 3.7×10^{10} independent Fab clones. The performance of the library was determined by the successful selection of on average 14 different Fabs against 6 antigens tested. These include tetanus toxoid, the hapten phenyl-oxazolone, the breast cancer-associated MUC1 antigen, and three highly related glycoprotein hormones: human chorionic gonadotropin, human luteinizing hormone, and human follicle-stimulating hormone. In the latter category, a panel of either hormone-specific or cross-reactive antibodies were identified. The design of the library permits the monitoring of selections with polyclonal phage preparations and to carry out large scale screening of antibody off-rates with unpurified Fab fragments on BIAcore. Antibodies with off-rates in the order of 10^{-2} to 10^{-4} s^{-1} and affinities up to 2.7 nM were recovered. The kinetics of these phage antibodies are of the same order of magnitude as antibodies associated with a secondary immune response. This new phage antibody library is set to become a valuable source of antibodies to many different targets, and to play a vital role in target discovery and validation in the area of functional genomics.

Display on filamentous phage in combination with selection forms a powerful tool for the identification of peptide- or protein-based drugs (1, 2). Of these, antibodies are especially of interest, due to their capacity to recognize a variety of targets with high specificity and affinity. In particular, the use of partially or completely human antibodies, which elicit no or minimal immune response when administered to patients, is yielding an increasing list of FDA-approved protein-based drugs (3). Phage display technology enables the generation of large repertoires of human antibodies (4–7), while the biopanning procedure permits the selection of individual antibodies with a desired specificity.

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Key to the success of the technology were two critical observations: (i) the expression of functional antibody fragments by secretion into the periplasm of *Escherichia coli* (8, 9), and (ii) the rapid access to variable region gene pools by the polymerase chain reaction (10–12). For the construction of antibody libraries, V-genes are amplified from B cell cDNA and heavy and light chain genes are randomly combined and cloned to encode a combinatorial library of single-chain Fv (scFv)¹ or Fab antibody fragments (4, 13–15). The natural primary (unselected) antibody repertoire within B cells contains a large array of antibodies that recognize a variety of antigens; this array can be cloned as a "naïve" repertoire of rearranged genes, by harvesting the V-genes from the IgM mRNA of B cells of unimmunized human donors, isolated from peripheral blood lymphocytes (4), from bone marrow or tonsils (7), or from similar animal sources (16). This procedure provides access to antibodies that have not yet encountered antigen, although the frequency of those genuine "germline" antibodies will depend heavily on the source of B cells (17). A single naïve library, if sufficiently large and diverse, can indeed be used to generate antibodies to a large panel of antigens, including self, non-immunogenic and relatively toxic antigens (4, 6). In a different approach, antibodies may be built artificially, by *in vitro* assembly of V-gene segments and D/J segments, yielding "synthetic" antibodies (5). A major drawback of these procedures is that from the initial naïve and synthetic libraries, only moderate affinity antibodies were isolated (4, 18). Over the last few years, more efficient techniques have been developed to build larger libraries of antibody fragments, using sophisticated *in vivo* recombination methods (6) or brute force cloning procedures (7, 19). Such large libraries have yielded a greater number of human antibodies per antigen tested, with on average much higher affinity (up to subnanomolar). However, technical restrictions on the size of libraries that may be obtained or handled in selection, the loss of library diversity upon library amplification, and the relatively long downstream analysis path of the selected antibodies, *i.e.* large scale affinity analysis, have limited the spread of these libraries as generic tools in antibody generation.

We describe here the generation of a very large antibody library based on the display of Fab fragments on phage. The choice for the Fab format was based on the notion that the monomeric appearance of the Fab should permit the rapid screening of large numbers of clones on kinetics of binding

¹ The abbreviations used are: scFv, single-chain Fv fragment; PCR, polymerase chain reaction; PBL, peripheral blood lymphocyte; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; RU, resonance units; hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; hFSH, human follicle-stimulating hormone; CTP, carboxyl-terminal peptide.

(off-rate) with crude protein fractions. Most large libraries made to date use the single-chain format (scFv) for display on phage (7, 19), but these fragments have the tendency to form dimers and higher order multimers in a clone-dependent and relatively unpredictable way (20–22). Multimeric antibody molecules bind more strongly to immobilized antigen than monomeric fragments because of their greater avidity, and therefore have higher “apparent” affinities (23). This explains why an accurate determination of the affinity is not easily possible with mixtures of mono- and multimeric scFv fragments. As a consequence, the affinity assay used for ranking individual clones (such as BIAcore analysis) often necessitates time-consuming purification to obtain the monomeric fraction of the selected antibody fragments (19, 24). An additional argument for the choice of the Fab format is to avoid possible problems with avidity of the displayed antibody fragment on the phage itself. The tendency of Fabs to be expressed at lower levels than scFv fragments and the lack of multimerization will lead to a lower display frequency and lower fraction of avid phage. The effect of multimerization of scFv on phage with respect to the selection of fragments with very low affinities has indeed been observed previously (6). Therefore, compared with scFv libraries, selections with Fab phage may be more governed by affinity rather than avidity, even when performing selections by panning on immobilized antigen (24) or with soluble multivalent antigen (25).

This report describes the strategy for the construction of a very large antibody library. An efficient cloning method, in which restriction fragments instead of PCR products were used, made it possible to generate a repertoire with as many as 37 billion different Fab clones. The performance of the library was analyzed by the selection with an extended panel of antigens including three closely related glycoprotein hormones, yielding a diverse set of specific antibody fragments for each antigen. Without using sophisticated selection protocols, hormone-specific as well as cross-reactive Fabs were retrieved against the highly homologous glycohormones, demonstrating that the library is a rich source of antibody specificities. The affinities of the anti-glycophormone antibodies varied between 2.7 and 38 nM. Finally, the Fab format indeed permitted the rapid screening and a reliable ranking of individual clones on off-rate using crude antibody fractions.

MATERIALS AND METHODS

RNA Isolation—As source of lymphoid tissues, we used peripheral blood lymphocytes from 4 healthy donors and part of a tumor-free spleen removed from a patient with gastric carcinoma. B lymphocytes were isolated from 2 liters of blood on a Ficoll-Paque gradient. For RNA isolation, the cell pellet was immediately dissolved in 50 ml of 8 M guanidinium thiocyanate, 0.1 M 2-mercaptoethanol (26). Chromosomal DNA was sheared to completion by passing through a narrow syringe (1.2/0.5-mm gauge), and insoluble debris was removed by low speed centrifugation (15 min at $2,934 \times g$ at room temperature). RNA was pelleted by centrifugation through a CsCl block gradient (12 ml of supernatant on a layer of 3.5 ml of 5.7 M CsCl, 0.1 M EDTA; in total four tubes) during 20 h at $125,000 \times g$ at 20 °C in a SW41 rotor (Beckman). The yield of total RNA was approximately 600 µg. RNA was stored at -20 °C in ethanol.

From the spleen, 2 g of tissue was used for homogenization with a Polytron homogenizer in 20 ml of 8 M guanidinium thiocyanate, 0.1 M 2-mercaptoethanol. The total volume was increased to 80 ml with guanidinium thiocyanate buffer, and after passage through a narrow syringe for shearing and removal of debris, RNA was pelleted as described before, except for 15 h at $85,000 \times g$ at 20 °C in a SW28.1 rotor (12 ml of supernatant on 3.5 ml of 5.7 M CsCl, 0.1 M EDTA in five SW28.1 tubes). From 2 g of tissue, 3 mg of total RNA was extracted.

Amplification of Variable Region Genes—Random primed cDNA was prepared with 250 µg of PBL RNA, while in a separate reaction 300 µg of spleen RNA was used as template. RNA was heat-denatured for 5 min at 65 °C in the presence of 20 µg of random primer (Promega); subsequently, buffer and dithiothreitol were added according to the

supplier's instructions (Life Technologies, Inc.), as well as 250 µM dNTP (Amersham Pharmacia Biotech), 800 units of RNasin (40 units/µl; Promega), and 2,000 units of Moloney murine leukemia virus reverse transcriptase (200 units/µl; Life Technologies, Inc.) in a total volume of 500 µl. After 2 h at 42 °C, the incubation was stopped by a phenol/chloroform extraction; cDNA was precipitated and dissolved in 85 µl of water.

Oligonucleotides used for PCR amplification of human heavy and light chain V-regions are described in Table I. IgM-derived heavy chain variable regions were obtained by a primary PCR with an IgM constant region primer. All primary PCRs were carried out with separate BACK primers and combined FOR primers, to maintain maximal diversity. The PCR products were reamplified with a combination of JHFOR primers, annealing to the 3' end of VH, and Sfi-tagged VHBACK primers, annealing to the 5' end, and subsequently cloned as VH fragments. The light chain V-genes of the κ and λ families were obtained by PCR with a set of CKFOR or CAFOR primer annealing to the 3' end of the constant domain and BACK primers, priming at the 5' end of the V-regions. The DNA segments were reamplified with primers tagged with restriction sites and cloned as V κ C κ and V λ C λ fragments.

PCR was performed in a volume of 50 µl using AmpliTaq polymerase (Cetus) and 500 pm of each primer for 28 cycles (1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C); nine separate IgM-derived VH amplifications were generated with 2 µl of random-primed cDNA (equivalent to 6 µg of PBL RNA or to 7 µg of spleen RNA) as template for each reaction. For the light chain families, 6 different V κ C κ products and 11 V λ C λ products (CA2 and CA7 primers combined in each reaction) were obtained. All products were purified from agarose gel with the QIAex-II extraction kit (Qiagen). As input for reamplification to introduce restriction sites, 100–200 ng of purified DNA fragment was used as template in a 100-µl reaction volume. The large amount of input, ensuring the maintenance of variability, was checked by analysis of 4 µl of the “unamplified” PCR mixture on agarose gel.

Construction of the Primary and Secondary Repertoires—For the construction of the primary heavy chain and the two primary light chain repertoires, the PCR products, appended with restriction sites, were gel-purified prior to digestion and the different VH, V κ , and V λ families combined into three groups. The V κ C κ and V λ C λ fragments were digested with *Apa*LI and *Asc*I, and cloned into the phagemid vector pCES1 (Fig. 1). The VH fragments, 1.5 µg in total, were digested with *Sfi*I and *Bst*EII and ligated in a 100–200-µl reaction mixture with 9 units of T₄-DNA ligase at room temperature to 4 µg, gel-purified vector pUC119-CES1 (similar to vector pCES1, but with the pIII gene deleted). The desalted ligation mixture for light or heavy chain pools was used for electroporation of the *E. coli* strain TG1, to create the one-chain libraries.

The Fab library was obtained by cloning of VH fragments, digested from plasmid DNA prepared from the heavy chain repertoires, into the plasmid collection containing the light chain repertoires. Plasmid DNA, isolated from at least 3×10^9 bacteria of the VH library, was digested with *Sfi*I and *Bst*EII for cloning in the vector that already contained λ and κ light chain libraries. To retain clones with internal *Bst*EII site in the V λ (this site is relatively frequent in some λ germline V-segments (27), and also in the constant domain of one of the λ families), the cloning of VHCH1 in the λ light chain repertoire containing vector was also carried out using *Sfi*I and *Not*I cloning sites, to create a less restriction-biased V λ library.

Selection of the Library—The rescue of phagemid particles with helper phage M13-KO7 was performed according to (4) on a 10-liter scale, using representative numbers of bacteria from the library for inoculation, to ensure the presence of at least 10 bacteria from each clone in the start inoculum. For selections, 10^{13} colony-forming units were used with antigens immobilized in immunotubes (Maxisorp tubes, Nunc) (4) or with soluble biotinylated antigens (28). The amount of the immobilized antigens tetanus toxoid and the hapten phenyl-oxazolone (conjugated to BSA in a ratio of 17 to 1) was reduced 10-fold during subsequent selection rounds, starting at 100 µg/ml at round 1. Capture with biotinylated antigen in solution was used for a 100-mer peptide encoding five copies of the tandem repeat of MUC1 (29), or with human chorionic gonadotropin (hCG), human luteinizing hormone (hLH), human follicle-stimulating hormone (hFSH) and its chimeric derivative (hFSH-CTP, containing the carboxyl-terminal peptide from the hCG β -subunit fused to the β -subunit of hFSH). Antigens were biotinylated at a ratio of 10–20 molecules of NHS-Biotin (Pierce) per molecule of antigen according to the supplier's recommendations. Unless stated otherwise, the antigens were used for selection at concentrations of 100, 30, and 10 nM during rounds 1, 2, and 3 respectively. For hFSH-CTP, 50, 15, and 10 nM was used, respectively; for MUC1 peptide 500, 100

TABLE I
Oligonucleotide primers used for construction of the library

A. Primary amplifications	
IgM heavy chain constant region	
HuIgMFOR	5'-TGG AAG AGG CAC GTT CTT TTC TTT-3'
κ light chain constant region	
Hu κ FOR	5'-ACA CTC TCC CCT GTT GAA GCT CTT-3'
λ light chain constant region	
Hu λ 2-FOR	5'-TGA ACA TTC TGT AGG GGC CAC TG-3'
Hu λ 7-FOR	5'-AGA GCA TTC TGC AGG GGC CAC TG-3'
V _H back	
HuVH1B/7A-BACK	5'-CAG RTG CAG CTG GTG CAR TCT GG-3'
HuVH1C-BACK	5'-SAG GTC CAG CTG GTR CAG TCT GG-3'
HuVH2B-BACK	5'-CAG RTC ACC TTG AAG GAG TCT GG-3'
HuVH3B-BACK	5'-SAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH3C-BACK	5'-GAG GTG CAG CTG GTG GAG WCY GG-3'
HuVH4B-BACK	5'-CAG GTG CAG CTA CAG CAG TGG GG-3'
HuVH4C-BACK	5'-CAG STG CAG CTG CAG GAG TCS GG-3'
HuVH5B-BACK	5'-GAR GTG CAG CTG GTG CAG TCT GG-3'
HuVH6A-BACK	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'
V _K back	
HuV κ 1B-BACK	5'-GAC ATC CAG WTG ACC CAG TCT CC-3'
HuV κ 2-BACK	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'
HuV κ 3B-BACK	5'-GAA ATT GTG WTG ACR CAG TCT CC-3'
HuV κ 4B-BACK	5'-GAT ATT GTG ATG ACC CAC ACT CC-3'
HuV κ 5-BACK	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'
HuV κ 6-BACK	5'-GAA ATT GTG CTG ACT CAG TCT CC-3'
V _L back	
HuV λ 1A-BACK	5'-CAG TCT GTG CTG ACT CAG CCA CC-3'
HuV λ 1B-BACK	5'-CAG TCT GTG YTG ACG CAG CCG CC-3'
HuV λ 1C-BACK	5'-CAG TCT GTC GTG ACG CAG CCG CC-3'
HuV λ 2-BACK	5'-CAR TCT GCC CTG ACT CAG CCT-3'
HuV λ 3A-BACK	5'-TCC TAT GWG CTG ACT CAG CCA CC-3'
HuV λ 3B-BACK	5'-TCT TCT GAG CTG ACT CAG GAC CC-3'
HuV λ 4-BACK	5'-CAC GTT ATA CTG ACT CAA CCG CC-3'
HuV λ 5-BACK	5'-CAG GCT GTG CTG ACT CAG CCG TC-3'
HuV λ 6-BACK	5'-AAT TTT ATG CTG ACT CAG CCC CA-3'
HuV λ 7/8-BACK	5'-CAG RCT GTG GTG ACY CAG GAG CC-3'
HuV λ 9-BACK	5'-CWG CCT GTG CTG ACT CAG CCM CC-3'

20, and 5 nm was used.

Screening and Sequencing of Clones—Soluble Fab was produced from individual clones as described before (4). Culture supernatants were tested in ELISA with directly coated antigen or indirectly captured biotinylated antigen via immobilized biotinylated BSA-streptavidin. Tetanus toxoid and pHx-BSA were coated at 10 μ g/ml in 0.1 M NaHCO₃, pH 9.6, for 16 h at 4 °C. For coating of hCG and hFSH-CTP, a concentration of 4 μ g/ml in 50 mM NaHCO₃, pH 9.6, was used. For capture of biotinylated antigens, biotinylated BSA was coated at 2 μ g/ml in PBS during 1 h at 37 °C. After 3 washes with PBS, 0.1% (v/v) Tween 20, plates were incubated during 1 h with streptavidin (10 μ g/ml in PBS/0.5% gelatin) (30). Following washing as above, biotinylated antigen was added for an overnight incubation at 4 °C at a concentration of 0.5 μ g/ml for MUC-1 peptide, 3 μ g/ml for hLH, and 0.6 μ g/ml for hFSH (binding to hCG was tested with directly coated antigen). The plates were blocked during 30 min at room temperature with 2% (w/v) semi-skim milk powder (Marvel) in PBS. The culture supernatant was diluted 1- or 5-fold in 2% (w/v) Marvel/PBS and incubated 2 h; bound Fab was detected with anti-myc antibody 9E10 (5 μ g/ml) recognizing the myc-peptide tag at the carboxyl terminus of the heavy Fd chain, and rabbit anti-mouse-HRP conjugate (Dako) (4). Following the last incubation, staining was performed with tetramethylbenzidine and H₂O₂ as substrate and stopped by adding 0.5 volume of 2 N H₂SO₄; the optical density was measured at 450 nm. Clones giving a positive signal in ELISA (over 2 times the background), were analyzed by *Bst*NI fingerprinting of the PCR products obtained by amplification with the oligonucleotide primers M13-reverse and geneIII-forward (4).

Large scale induction of soluble Fab fragments from individual clones was performed on a 50-ml scale in 2 \times TY containing 100 μ g/ml ampicillin and 2% glucose. After growth at 37 °C to an OD₆₀₀ of 0.9, the cells were pelleted (10 min at 2,934 \times g) and resuspended in 2 \times TY with ampicillin and 1 mM isopropyl-1-thio- β -D-galactopyranoside. Bacteria were harvested after 3.5 h of growth at 30 °C by centrifugation (as before); periplasmic fractions were prepared by resuspending the cell pellet in 1 ml of ice-cold PBS. After 2–16 h of rotating head-over-head at 4 °C, the spheroplasts were removed by two centrifugation steps; after spinning during 10 min at 3,400 \times g, the supernatant was clarified by an additional centrifugation step during 10 min at 13,000 \times g in an

Eppendorf centrifuge. The periplasmic fraction obtained was directly used for determination of fine specificities by surface plasmon resonance or for Western blot studies (described below).

For sequencing, plasmid DNA was prepared from 50-ml cultures grown at 30 °C in LB-medium, containing 100 μ g/ml ampicillin and 2% glucose, using the Qiagen Midi-kit (Qiagen). Sequencing was performed with the thermocycling kit (Amersham Pharmacia Biotech) with CY5-labeled primers CH1FOR (5'-GTC CTT GAC CAG GCA GCC CAG GGC-3') and M13REV (5'-CAG GAA ACA GCT ATG AC-3'); samples were run on the ALF-Express (Amersham Pharmacia Biotech). V-gene sequences were aligned to V-BASE or the Sanger Center.²

Determination of Fine Specificities of the Anti-hormone Fabs by Western Blot and Surface Plasmon Resonance—An hCG preparation purified from urine and immuno-affinity-purified recombinant hLH, hFSH, and hFSH-CTP produced in CHO cells (30, 31) were used for Western blot studies as described elsewhere (32). Between 0.5 and 1 μ g of each hormone was loaded per lane; proteins were diluted in non-reducing sample buffer and boiled during 5 min or directly applied on gel without heat treatment; proteins were transferred to blotting membrane by electrotransfer. Blots were subsequently incubated for 16 h at room temperature with a 10-fold diluted periplasmic fraction in PBS, 4% Marvel. Bound Fab was detected with anti-myc antibody 9E10 (5 μ g/ml) and 4,000-fold diluted anti-mouse alkaline phosphatase-conjugate (Promega), using the substrates 5-bromo-1-chloro-3-indolyl phosphate and nitro blue tetrazolium (Roche Molecular Biochemicals) for visualization.

The specificity of the Fabs was further characterized by surface plasmon resonance (BIAcore 2000, Biacore). Recombinant hLH, hFSH, and the urinary hCG were immobilized on the flow cells of a CM chip using the NHS/EDC kit (Biacore AB, Uppsala, Sweden), yielding a surface of 1906 RU for hLH, 1529 RU for hFSH, and 1375 RU for hCG.

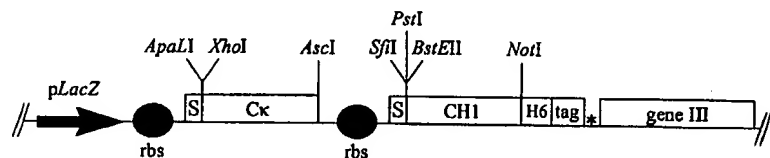
² V-BASE is available via the World Wide Web (Medical Research Council Center for Protein Engineering, 1997; <http://www.mrc-cpe.cam.ac.uk/int-doc/public/INTRO.html>); Sanger Center is also available via the World Wide Web (Sanger Center Germline Query, 1997; <http://www.sanger.ac.uk/Data/Search/germline-search.html>).

TABLE I—continued

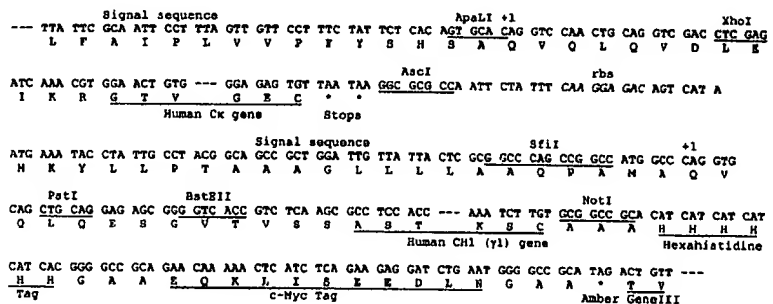
B. Secondary amplifications	
κ light chain constant region	
HuCκ-FOR-ASC	5'-ACC GCC TCC ACC GGG CGC GCC TTA TTA ACA CTC TCC CCT GTT GAA GCT CTT-3'
λ light chain constant region	
Huλ2-FOR-ASC	5'-ACC GCC TCC ACC GGG CGC GCC TTA TTA TGA ACA TTC TGT AGG GGC CAC TG-3'
Huλ7-FOR-ASC	5'-ACC GCC TCC ACC GGG CGC GCC TTA TTA AGA GCA TTC TGC AGG GGC CAC TG-3'
V _H back	
HuVH1B/7A-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG RTG CAG CTG GTG CAR TCT GG-3'
HuVH1C-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTR CAG TCT GG-3'
HuVH2B-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTG GAG TCT GG-3'
HuVH3B-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTG GAG TCT GG-3'
HuVH3C-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTG GAG TCT GG-3'
HuVH4B-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTG GAG TCT GG-3'
HuVH4C-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTG GAG TCT GG-3'
HuVH5B-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTG GAG TCT GG-3'
HuVH6A-BACK-SFI	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTG GAG TCT GG-3'
V _H forward	
HuJH1/2-FOR	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
HuJH3-FOR	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJH4/5-FOR	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJH6-FOR	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'
Vκ back	
HuVκ1B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAC ATC CAG WTG ACC CAG TCT CC-3'
HuVκ2-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAT GTT GTG ATG ACT CAG TCT CC-3'
HuVκ3B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAA ATT GTG WTG ACR CAG TCT CC-3'
HuVκ4B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAT ATT GTG ATG ACC CAC ACT CC-3'
HuVκ5-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAA ACG ACA CTC ACG CAG TCT CC-3'
HuVκ6-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT GAA ATT GTG CTG ACT CAG TCT CC-3'
Vλ back	
HuVλ1A-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG TCT GTG CTG ACT CAG CCA CC-3'
HuVλ1B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG TCT GTG YTG ACG CAG CCG CC-3'
HuVλ1C-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG TCT GTC GTG ACG CAG CCG CC-3'
HuVλ2-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAR TCT GCC CTG ACT CAG CCT-3'
HuVλ3A-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT TCC TAT GWG CTG ACT CAG CCA CC-3'
HuVλ3B-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT TCT TCT GAG CTG ACT CAG GAC CC-3'
HuVλ4-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAC GTT ATA CTG ACT CAA CCG CC-3'
HuVλ5-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG GCT GTG CTG ACT CAG CCG TC-3'
HuVλ6-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CTT AAT TTT ATG CTG ACT CAG CCC CA-3'
HuVλ7/8-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CAG RCT GTG GTG ACY CAG GAG CC-3'
HuVλ9-BACK-APA	5'-ACC GCC TCC ACC AGT GCA CWG CCT GTG CTG ACT CAG CCM CC-3'

FIG. 1. Phagemid vector pCES1 for display of antibody Fab fragments. Schematic representation (A) and polylinker region (B) of pCES1. The polylinker region comprises two signal sequences (S; pelB and the gene III leader sequence), the Cκ domain, ribosome binding site (rbs), CH1 domain, hexahistidine tag (H6), and a c-myc-derived sequence (tag). Variable domain genes can be cloned as *Apa*LI-*Xho*I or *Apa*LI-*Asc*I fragments (for VL or VLCL, respectively) and *Sfi*I/*Pst*I-*Bst*EII or *Sfi*I-*Not*I fragments (for VH or VHCH1, respectively). The amber stop codon (*) between the antibody genes and bacteriophage gene III enables the production of soluble Fab fragments in a non-suppressor strain of *E. coli*. Expression of the bicistronic operon is under control of the *LacZ* promoter (*pLacZ*).

A



B



Periplasmic fractions were diluted 3-fold in Hepes-buffered saline (10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.05% (v/v) surfactant P20, pH 7.4) and analyzed using a flow rate of 10 μ l/min.

Purification of Soluble Fab Fragments—Fabs were obtained by refolding of the total bacterial proteins from a 50-ml culture (33). Briefly, the pelleted cells from a 50-ml induced bacterial culture were resuspended in 8 ml of 8 M urea (in PBS). After sonication, the mixture was

rotated head over head for 30 min and insoluble material was removed by centrifugation for 30 min at 13,000 \times g. The supernatant was dialyzed against PBS with four buffer changes. Insoluble proteins were removed by centrifugation and the flow-through fraction, obtained by filtration through a 0.2- μ m membrane, was immediately loaded on an hCG column (bed volume 0.3 ml). The column material was prepared by coupling 8.4 mg of protein to 1 g of Trarvl-Senhamse according to the

supplier's instructions (Pierce). The column (1 ml column material) was washed with 10 volumes of 100 mM Tris, 500 mM NaCl, pH 7.5; then subsequently with 10 volumes of 100 mM Tris, 500 mM NaCl, pH 9.5; and 2 volumes of 0.9% NaCl. Bound Fab was eluted in a batchwise fashion with 2 volumes of 0.1 M triethylamine. After a 10-min incubation, the effluent was collected and immediately neutralized with 0.5 volume of 1 M Tris, pH 7.5. The Fab fraction was dialyzed against PBS using a Microcon 30 spin dialysis filter (Amicon). Finally, a gel filtration analysis was carried out on a Superdex 75HR column (Amersham Pharmacia Biotech). The yield was determined by measuring the optical density at 280 nm (using a molar extinction coefficient of 13 for Fabs).

Determination of On- and Off-rate Using Surface Plasmon Resonance with Crude Fab Preparations—The kinetics of binding were analyzed by surface plasmon resonance on three different hCG surfaces (303, 615, and 767 RU immobilized, with 4955 RU of BSA on a separate flow cell as a negative control). Obviously, ranking of the off-rates of the individual clones needs to be done by analysis with the BIAevaluation software. Fab present in crude periplasmic extracts was quantified on a high density surface of purified anti-human Fab polyclonal antibody (Pierce) as described (34). Anti-hCG Fabs controls were purified by affinity chromatography on hCG columns as described above and used to calibrate the system.

RESULTS

Design of the Non-immunized Phage Antibody Library—We considered a number of variables to address in the construction of a novel, very large phage antibody library: (i) the primer design was optimized for amplification of variable gene pools to maintain maximum diversity; (ii) a highly efficient two-step cloning method was developed to obtain a very large non-immunized library; (iii) an antibody format and compatible cloning vector were chosen, which should permit the rapid downstream analysis of selected clones.

In order to achieve access to as many different human heavy and light chain V-region gene segments as possible, a new set of oligonucleotide primers was developed (Table I), the design of which was based on the most recent sequence information provided by the V-base (see "Materials and Methods"). The primers should allow efficient amplification of all commonly used V-gene segments. Further, to obtain large sized libraries (over 10^{10} in diversity), we used a two-step cloning procedure; heavy and light chain variable genes were first separately cloned as digested PCR products, and then combined by restriction fragment cloning to form a large library of Fab fragments. This cloning procedure should be a more efficient route for library construction than the relatively inefficient direct cloning of digested PCR products, while avoiding the DNA instability often associated with *in vivo* recombination systems (35).

As choice of antibody format, we preferred the Fab above the scFv format, because this would allow us to develop rapid high through-put affinity-screening assays for crude antibody preparations. Many scFv fragments indeed form higher molecular weight species including dimers (20, 21) and trimers (36), which complicate both selection and characterization. We chose the Fab display format, in which the heavy chain is linked to the phage coat protein pIII, and also carries a tag for detection and purification (see below). The light chain is expressed as separate fragment, secreted into the periplasm, where it can pair with the heavy chain (37).

To incorporate all these improvements, a new phagemid vector, pCES1 (Fig. 1), was constructed, which allows the stepwise cloning of antibody fragments in Fab format. In this vector system, the variable heavy chain region genes are cloned as VH-gene fragments; the vector supplies all Fabs with a human gamma-1 CH1 gene. The Fd fragment is fused to two tags for purification and detection (a histidine tail for immobilized metal affinity chromatography (38) and a c-myc-derived tag (39)), followed by an amber stop codon (37) and the minor coat protein III of filamentous phage fd. The antibody light chain is

TABLE II
Size and composition of the phage antibody libraries

Source B-cells	Library	Insert %	Size ^a cfu
One-chain libraries	VH	64	1.8×10^8
	V κ	100	5.7×10^7
	V λ	96	4.5×10^7
Spleen	VH	75	8.0×10^7
	V κ	100	3.0×10^7
	V λ	80	1.0×10^7
Fab library	PBL	90	1.95×10^{10}
	Spleen	80	2.35×10^{10}
Total size		86	4.3×10^{10}

^a Not corrected for clones without Fab encoding insert. cfu, colony-forming units.

TABLE III
Overview of results of selections with a diverse set of antigens

Antigen	ELISA positives			Different Ag-specific clones
	Round 2	Round 3	Round 4	
TT ^a	13/36	69/80		>21
phOx ^b	29/37	70/80		>24
MUC1 ^c	1/37	32/87	25/87	14
hCG ^d		24/48	34/48	8
hLH ^e		40/45	30/45	>21
hFSH ^f		30/45	40/45	6
hFSH-CTP ^g		16/48	17/48	7

^a TT, tetanus toxoid.

^b phOx, 2-phenyloxazol-5-one.

^c MUC1, mucin-1-derived peptide.

^d hCG, human chorionic gonadotropin.

^e hLH, human luteinizing hormone.

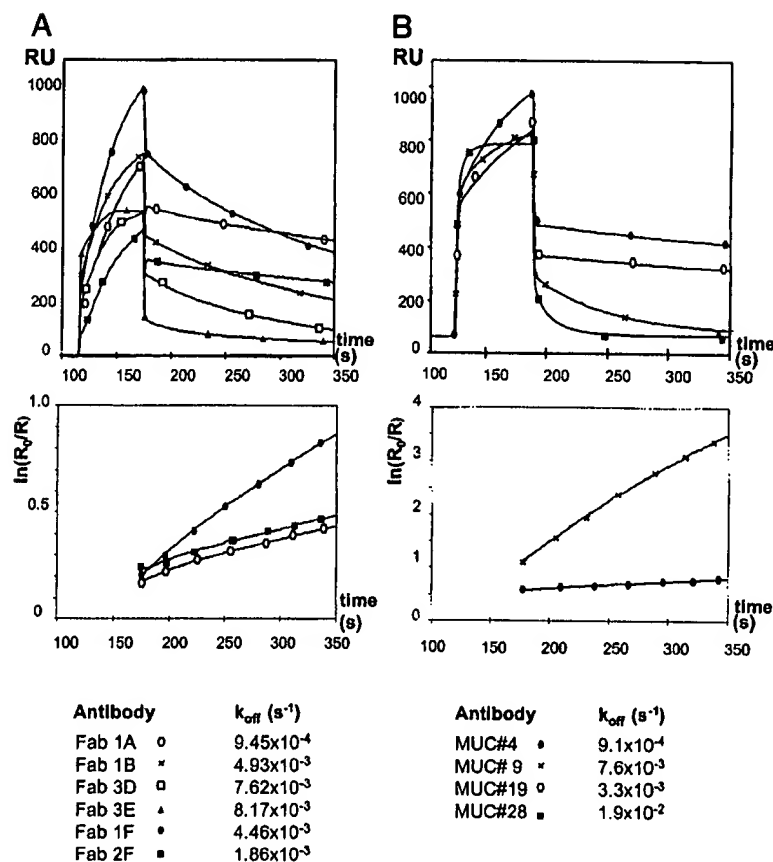
^f hFSH, human follicle-stimulating hormone.

^g hFSH-CTP, chimeric product of β -CTP of hCG fused to β -FSH.

cloned as full VLCL fragment, for directed secretion and assembly with the VHCH1 on the phage particle.

Library Construction—The Fab library was constructed in two steps. In the first step, variable region gene pools were amplified from approximately 4×10^8 B cells from the PBLs of four healthy donors, and, as a source of possibly more heavily mutated IgM antibodies, from a segment of a (tumor-free) spleen removed from a patient with gastric carcinoma, containing approximately 1.5×10^8 B cells (40). Only IgM-derived VH segments were amplified by using an amplification with an oligonucleotide primer located in the first constant domain of this isotype. These products were cloned into phagemid vector pCES1 for VL, and in pUC119-CES1 for VH (cloning was more efficiently in the smaller sized vector, in which gene III was deleted). The PBL- and spleen-derived VH, V κ , and V λ libraries were cloned separately to maintain diversity, to yield one-chain libraries in size typical for libraries made by cloning of PCR fragments (4): 1.75×10^8 individual clones for the heavy chain, 9.4×10^7 clones for V κ , and 5.2×10^7 clones for V λ . An overview is given in Table II. In the second step, the heavy chain fragments were digested from plasmid DNA isolated from the primary VH repertoire, and cloned into the vector containing the light chain repertoires (again separately for PBL- and spleen-derived repertoires; Table II). The libraries were combined using this efficient cloning procedure, to create a non-immunized Fab repertoire with 3.7×10^{10} individual clones (4.3×10^{10} recombinant clones, 86% of which have a full-length Fab insert), with 70% of clones harboring a κ light chain, 30% a λ chain. All of 20 clones with full-length Fab insert

FIG. 2. Off-rate screening in BIAcore of selected tetanus toxoid and MUC1 binding Fab fragments. Periplasmic fractions from four anti-tetanus toxoid clones (A) and from anti-MUC1 clones (B) were injected at $t = 115$ s on flow cells with immobilized tetanus toxoid and MUC1 peptide, respectively. At $t = 175$ s, the dissociation phase started by washing with Hepes-buffered saline buffer. Below the sensorgram, the derivative $\ln(R_0/R)$ of two representative clones was plotted against the time (R_0 taken from $t = 175$ s), which should be linear for a truly monophasic dissociation. Resulting dissociation rates are shown below each series of sensorgrams.



tested scored positive in dot-blot analysis with the 9E10 antibody indicating an expression level of soluble Fab of at least 0.2 mg/liter.

Quality Control of the Library by Selection with a Panel of Antigens—We evaluated the library by selection with different antigens, the screening data of which are summarized in Table III. First, the results from three model antigens, the protein tetanus toxoid, the hapten 2-phenyloxazol-5-one (phOx) (41), and the peptide MUC1, are discussed. Three rounds of biopanning on tetanus toxoid yielded a diverse set of ELISA-positive Fabs; in a series of 47 tetanus toxoid binding Fabs, at least 21 were different with regard to *Bst*NI fingerprint. Similarly, an extensive panel of phOx-specific Fabs was retrieved after three rounds of panning; at least 24 different clones were identified in a series of 50 ELISA-positive clones. Solution capture with biotinylated MUC1 peptide resulted in the selection of 14 different antibody fragments out of 37 ELISA-positive clones selected after 3 rounds (Table III).

Rapid Dissociation Rate Determination—With such large panels of antibodies isolated, it is crucial to have methods available to readily determine the kinetic parameters of each individual antibody-antigen interaction. Such an assay should be robust and ideally employ non-purified antibody fragments. We tested whether it would be feasible to use periplasmic fractions prepared from small scale cultures for a rapid and accurate determination of the off-rate of the antibodies using surface plasmon resonance. An example of an overlay plot with the sensorgrams from a series of tetanus toxoid-specific Fabs is shown in Fig. 2. The plot of $\ln(R_0/R)$ versus time (Fig. 2, lower graphs) reveals a linear relation with slope k_d (off-rate), thereby confirming a monophasic dissociation, which can be expected for a truly monomeric Fab fragment binding to a low density antigen surface. At the beginning of the dissociation

phase, the relation is not linear due to a difference in composition of the BIAcore running buffer (Hepes-buffered saline) and the buffer solution of the Fab samples (phosphate-buffered saline); this may be avoided by pre-dialysis. Using this off-rate screening assay, we determined the off-rates for the best tetanus toxoid- and MUC1-specific Fabs to be in the order of 10^{-2} to $10^{-4} s^{-1}$ (Fig. 2).

Selection of Fab Antibodies against Related Glycoprotein Hormones—As a more stringent test panel of antigens to assay the performance of the library, we chose to derive antibodies to three structurally related glycoproteins: hCG, hLH, and hFSH (reviewed in Ref. 42). These hormones are heterodimers sharing an identical α -chain with 92 amino acid residues, but have β -subunits of different composition and length. The β -chain of hCG contains 145 amino acid residues, and the one from hLH only 121 residues, the latter showing 85% homology to β -hCG. The β -chain of hFSH is only 111 amino acids and shares 36% of the residues with hCG. Antibodies that specifically detect hCG have been used extensively in pregnancy tests (42) and for cancer diagnosis (43, 44). A large set of antibodies to these targets would extend the limited number of hormone-specific antibodies (especially against hLH), obtained using the hybridoma technology (42). The human origin of the antibodies might be beneficial when using these for imaging or therapy of testicular and bladder cancer (43, 44).

Selections were thus performed on biotinylated urinary hCG, recombinant hLH, hFSH, and hFSH-CTP (the latter is a chimeric molecule containing the carboxyl-terminal peptide of β -hCG fused to the β -chain of FSH; Ref. 45). The highest degree of enrichment with respect to the increase in the number of eluted phage particles in round 3 versus round 1 was found for hCG (10,000-fold), followed by hFSH-CTP (1,000-fold), hFSH (300-fold), and hLH (150-fold). Polyclonal phage of selected

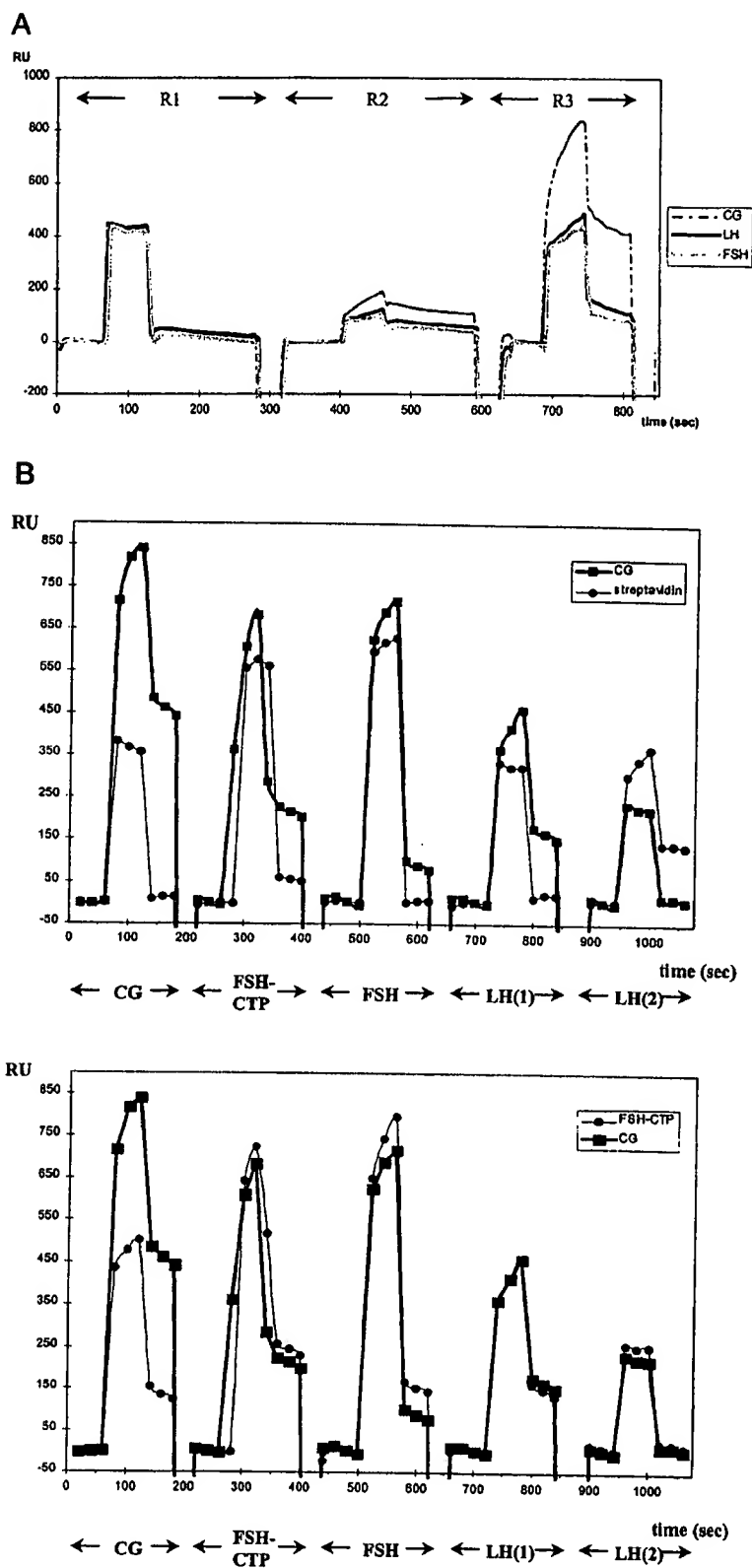


FIG. 3. Monitoring of selections with polyclonal phage using surface plasmon resonance. Polyclonal phage populations from rounds 1, 2, and 3 (*R1*, *R2*, and *R3*, respectively) of the selection with hCG, were analyzed on flow cells with hCG, hLH, and hFSH; at $t = 70$ s, $t = 400$ s, and $t = 690$ s, phage was injected, ending at $t = 120$ s, $t = 450$ s, and $t = 740$ s, respectively (A). Analysis of phage from round 3 selected with the antigens hCG, hFSH-CTP, hFSH, and hLH (the latter was selected with 100 nM hormone (coded *LH(1)*) or 10 nM (coded *LH(2)*) at round 1), using flow cells with hCG and streptavidin (upper sensorgram) or with hFSH-CTP, and hCG (lower sensorgram); injection was started at $t = 60$ s, $t = 270$ s, $t = 500$ s, $t = 720$ s, and $t = 950$ s, and terminated at $t = 120$ s, $t = 330$ s, $t = 560$ s, $t = 780$ s, and $t = 1010$ s, respectively (B).

populations were tested for binding using sensor chips containing immobilized hormones (46). Polyclonal phage selected on hCG showed binding after two and three rounds of selection to all three proteins, i.e. hCG, hLH, and hFSH, with the strongest signal visible for hCG (Fig. 3A); after three selection rounds, approximately 400 RU of bound material is visible at the start

of the dissociation phase (the large peaks visible during the first phase of association and dissociation are caused by refractive index changes due to buffer effects). Similar analysis of the polyclonal phage populations selected for three rounds on hFSH showed a dominance of hFSH-specific binding approx. 150 RU while selections on hFSH-CTP yielded binding to both

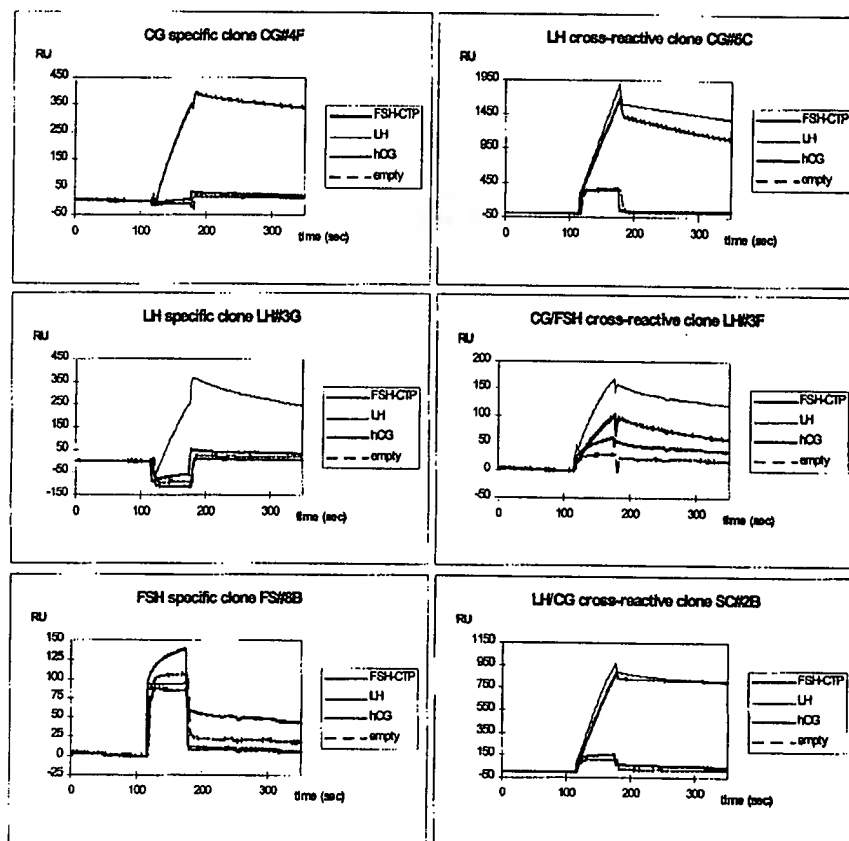


FIG. 4. Analysis of specificity of selected anti-glycoprotein hormone Fabs using surface plasmon resonance. Periplasmic fractions from clones CG#4F, CG#5C, LH#3G, LH#3F, FS#8B, and SC#2B were injected at $t = 120$ s on flow cells with immobilized hFSH-CTP, hLH, and hCG, and on an empty surface; at $t = 180$ s, the injection phase was stopped and the cells were washed with Hepes-buffered saline buffer.

hFSH and hCG (250 RU, Fig. 3B). In this case, the control surface was coated with streptavidin; no specific signals were obtained (<50 RU between the signal before and after injection of the phage preparation). Selections on hLH yielded antibodies reactive with hFSH and hCG (thus most likely anti- α -chain antibodies; marked LH (1) in Fig. 3B). When hLH was used at lower concentrations (at 10 nM in round 1 and 3 nM during the subsequent selection rounds), a signal was seen with streptavidin only (marked LH(2) in Fig. 3B), due to the selection of streptavidin-specific antibodies. Thus, this polyclonal phage screening provides a rapid test to check the overall quality of the clones in the selected repertoire, and may also be used to guide the choice of the conditions for the next selection round (46).

Specificity Analysis of the Selected Monoclonal Fabs—ELISA of monoclonal phage antibodies revealed that three rounds of selection with hCG indeed resulted in the isolation of a high percentage (74%) of clones positive for the gonadotropin. 27% of these clones were hLH-cross-reactive; none were reactive against streptavidin. *Bst*NI fingerprint analysis of the ELISA-positive clones revealed a high degree of diversity (8 different patterns). From a representative hCG-specific (coded CG#4F) and hLH-cross-reactive (CG#5C) clone, the specificity was tested in BIAcore using unpurified soluble Fab fragments (Fig. 4). Clone CG#4F gave a high response on hCG, with no visible binding to either hLH or hFSH-CTP. In contrast, clone CG#5C bound to hCG and hLH, but not to hFSH-CTP. Western blots, with the different hormones in non-reduced form, showed the specific recognition of the β -subunit of hCG by clone CG#4F, while the cross-reactive clone CG#5C reacted with the β -subunit of both hCG and hLH (Fig. 5).

Selection with the hormone hLH resulted in the isolation of hLH-specific and hCG-cross-reactive clones. Examination of individual clones from selection round three in ELISA revealed

a large fraction of hLH-specific clones (69%), and a minor group of cross-reactive clones (16%); no streptavidin-reactive clones were selected. Within the group of specific clones, a large array of different species (>21) could be discriminated by fingerprint analysis; however, all cross-reactive species had a single pattern. The unique hLH specificity was confirmed for representative clones LH#2H and LH#3G, shown in surface plasmon resonance (shown for clone LH#3G in Fig. 4); and on Western blot (illustrated for clone LH#3G in Fig. 5). This Fab only recognizes the intact α/β -heterodimer of hLH. Two representative clones of a pan-reactive antibody in ELISA, coded LH#1C and LH#3F, reacted in BIAcore with hFSH-CTP, hCG, and hLH (shown for clone LH#3F in Fig. 4), and in Western blot analysis with the α -chains from all three hormones (data not shown).

When hFSH was used as antigen during selection, six different antibodies were isolated from the library, with one type, represented by clone FS#8B, dominating the selected population. This Fab only recognized hFSH in BIAcore (Fig. 4), and, as Western blot analysis demonstrated, in particular its β -unit (Fig. 5). Further, the specificity of an α -chain binding clone, SC#2B, was confirmed in BIAcore (Fig. 4) and Western blot (Fig. 5).

Upon selection with FSH-CTP, seven different α -chain-specific Fabs were identified by fingerprint analysis, from which the clones coded SC#2B, SC#2F, SC#2G, and SC#4G were examined in more detail. Immunoblot analysis with the recombinant Fab as detecting antibody confirmed the α -chain specificity (blot incubated with clone SC#2B is shown in Fig. 5).

Phage-selected Antigen-specific Clones Are Intact Fab Fragments—There have been some reports on the isolation from scFv or Fab libraries of antigen-specific single-domain or other artificial antibody fragments (47, 48). Therefore, we tested the integrity of the selected Fabs. First the nature of the Fab

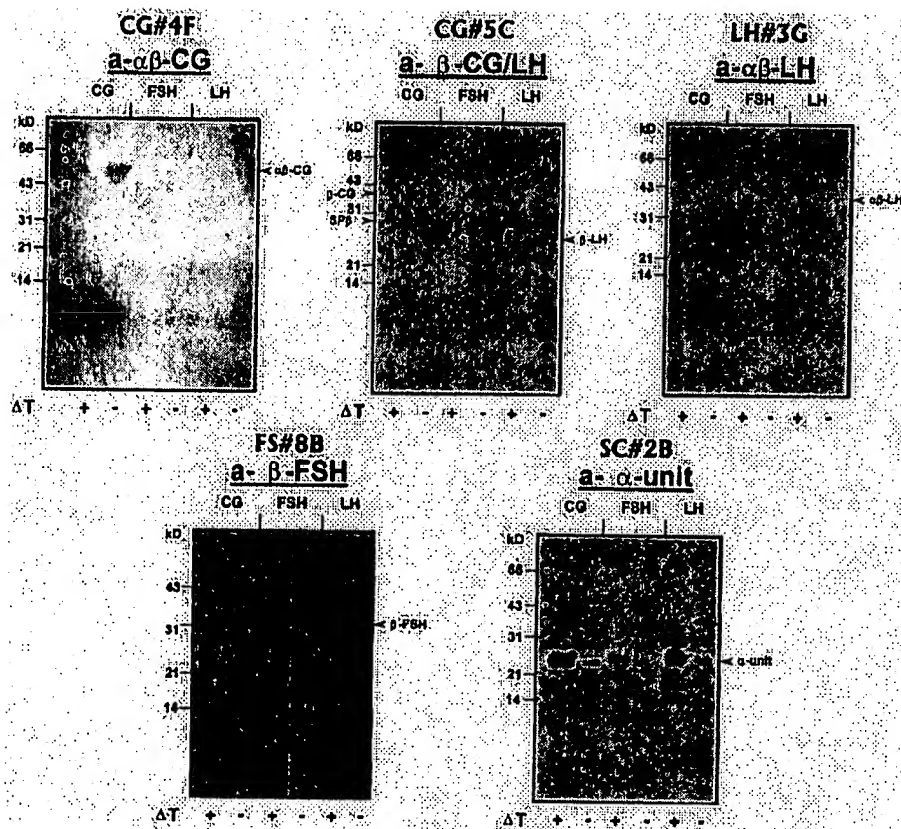


FIG. 5. Specificities of the Fabs determined with Western blot analysis. The glycoprotein hormones hCG, hFSH, and hLH were diluted in non-reducing sample buffer, and boiled (ΔT) or not heat-treated (T), loaded on a 15% SDS-PAGE, blotted onto nitrocellulose filter, and detected with the indicated Fabs. The molecular forms of the hormones recognized by the Fabs of clones CG#4F, CG#5C, LH#3G, FS#8B, and SC#2B are indicated with arrows.

fragments in periplasmic fractions was determined in Western blot. When incubated in non-reducing sample buffer, two products were detected with the 9E10 antibody, which recognizes the myc-tag at the end of the CH1 domain (Fig. 6A); the major product is the intact Fab molecule, in which an intermolecular disulfide bridge covalently links heavy and light chain fragments; the low molecular product is most likely derived from non disulfide bridge linked heavy chains. Analysis with anti-light chain sera reveals a similar pattern and shows that the clones use a nearly equal percentage of κ and λ chains (found in 6 and 7 clones, respectively, of a total of 13 tested) (Fig. 6, B and C). From the densities of the blots, it would be unlikely that all of the light chain would be complexed as Fab. Instead, it appears that in many cases more light than heavy chain fragment is produced, which may be expected from the design of the bicistronic expression cassette. Upon purification of functional antigen-binding fragments using denaturation and refolding, followed by affinity chromatography, this excess of light chain disappears, as expected (shown with a Coomassie blue-stained protein gel, for five clones in Fig. 7). Upon reduction, equal amounts of heavy and light chain are seen, while under non-reducing conditions (shown for one clone only), the main product is represented by the disulfide linked Fab-molecule, with an equal amount of the (most likely) non-covalently linked VHCH1 and VLCL products visible. Production yields of selected hormone-specific Fabs varied between 160 μ g and 1.43 mg of Fab/liter of culture, which was in the same range as was found for the unselected Fabs (data not shown).

Use of Diverse Germline Sequences—A panel of 14 antigen-specific Fabs was fully sequenced (Table IV; 3 anti-MUC1 antibodies positive in BIAcore on 100-mer peptide, and 11 anti-gonadotropin antibodies). The heavy chain genes are derived from the four largest VH families (VH1, VH3, VH4, and

VH6); the VL genes belong to one of four V κ families or one of three V λ families. Chain promiscuity is seen for the α -chain-specific clone SC#4G, the α/β -LH-specific clones LH#2H and LH#3G, and β -FSH-specific clone FS#8B, which all used a highly homologous V κ 2 light chain gene segment (A19, previously coded DPK15) combined with different heavy chain fragments. This promiscuity for A19 was previously found in antibodies derived from a synthetic Fab repertoire (35). The three anti-MUC1 antibodies use heavy and light chain genes derived from two different VH and V κ families; clone MUC#9 uses a VH with a cross-over of two segments. It is remarkable that both MUC#4 and MUC#9 VH genes use the same reading frame of the same D-segment (D6–13; with a stretch of 13 or 16 bp from this segment, respectively; Ref. 49), encoding an alanine-glycine stretch (AAAG; Table IV). This may reflect a similar mode of binding to MUC1, despite the use of a different light chain by these two clones.

Measurement of Affinities with Purified Anti-hCG Fabs—The affinities and off-rates of affinity-purified hCG-reactive Fabs LH#1C, SC#2B, LH#3F, and CG#5C were determined. The off-rates for most Fabs were in the order of 10^{-2} and 10^{-3} s $^{-1}$ (Table V). The off-rate values obtained using crude periplasmic fractions were in good agreement with the values found for the purified Fabs, validating the utility of the off-rate screen with unpurified Fab fragments. The affinities, 23 and 38 nM for the α -subunit-specific antibody LH#1C and the β -subunit hCG/hLH-cross-reactive antibody CG#5C, respectively, are comparable to the affinity of antibodies selected from a murine immune phage antibody library³; the top affinity, 2.7 nM for the α -chain-specific Fab SC#2B (Table IV), approaches the values

³ H. J. de Haard and R. Kozemier, unpublished results.

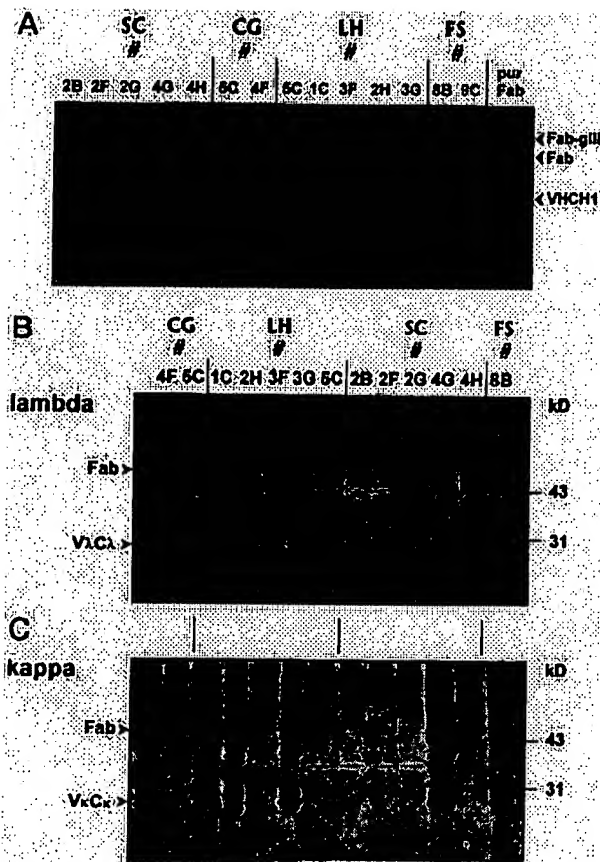


FIG. 6. Examination of the integrity of produced Fab fragments on Western blot. The periplasmic fractions from the indicated glycoprotein-specific Fab clones were boiled in non-reducing sample buffer and loaded on 12.5% SDS-PAGE. The blots were incubated with the anti-myc mAb 9E10 (A), anti-human λ polyclonal antibodies (upper panel of B), or with anti-human kappa polyclonal antibodies (C). Affinity-purified Fab was used as a control on the blot incubated with anti-myc mAb (indicated with *pur Fab*).

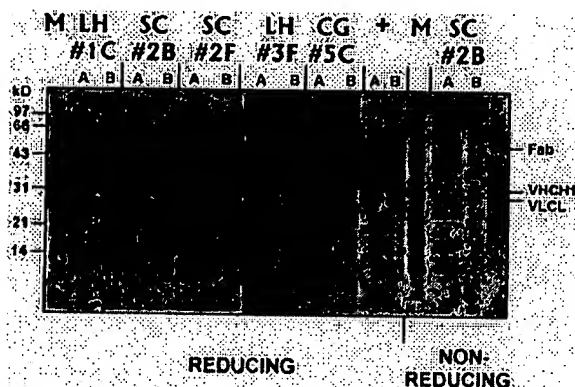


FIG. 7. SDS-PAGE of affinity-purified Fabs. hCG binding Fabs produced by clones LH#1C, LH#3F, CG#5C, SC#2B, and SC#2F were purified by affinity chromatography on hCG-Sepharose, and analyzed on a Coomassie-stained 12.5% SDS-PAGE gel under reducing conditions, and for Fab SC#2B also under non-reducing conditions. As positive control, a Fab fragment made by proteolytic digestion of a human monoclonal antibody was included (marked with +). B is a 4-fold dilution of A of the same sample.

of the best anti-hCG monoclonal antibodies.³

DISCUSSION

This report describes the construction of a phage display library from the *in vivo* rearranged V-gene repertoire of human

donors and its evaluation by selection with a panel of hapten and protein antigens. The source of antibody producing B cells was twofold: peripheral blood lymphocytes, which are mainly IgM-positive cells, and B cells from a human spleen. The theoretical diversity of a combinatorial antibody library made from the PBLs of one donor is much larger than what can be practically made or accessed (10^{14} combinations with 10^7 individual B cells). However, there may be a strong bias in the diversity introduced because of the donor's recent immune history and major differences in mRNA contents and clonal outgrowth. Therefore, in previously constructed very large non-immunized libraries, B cells from many different donors were used (7). Most probably the repertoire will be limited in diversity by using random priming because plasma cells (mostly of the IgG type) will produce 10,000-fold more mRNA when compared with non-activated B cells; a better source for non-immunized libraries are IgM primed V-genes (4). We have successfully used B cells from only a few donors (four for the PBLs and one spleen), but have aimed to access a more diverse pool (spleen and PBL-IgM in this library, *versus* tonsil and PBL random primed V-genes). In the PBL pool of adults, over 60% of the B cells are unmutated IgM+IgD+ naive B cells, while the remaining cells are memory cells that have acquired somatic mutations (50). Since the latter class contains more mRNA (17), most of the VH genes derived from cloning of this pool are expected to carry mutations. Similarly the IgM pool in spleen B cells will contain antibodies with mainly mildly mutated germline genes. Indeed, many of the selected antibodies carry a low level of mutations in the heavy chain genes. Some antibodies (*i.e.* clone LH#3G) are nevertheless completely germline encoded and yet of high affinity and specificity (similarly to what has been described for some B cell-derived antibodies (51)). There may be sources of B cells that will yield a truly naive V-gene repertoire (possibly, bone marrow-derived and/or IgM+IgD+CD27- B cells), but it remains to be seen if these V-gene sources will also yield better libraries. Indeed, when comparing synthetic antibody libraries (35), which incorporate germline encoded V-gene segments with non-immunized human V-gene libraries such as the one presented here, it is difficult to pinpoint any performance differences with regard to affinity and specificity of selected antibodies.

We employed an efficient two-step cloning procedure with DNA fragments digested from plasmid DNA instead of PCR fragments, to obtain the largest non-immunized human Fab repertoire reported to date, with a theoretical diversity of 37 billion different clones.

The choice of the Fab format was based on the possibility to develop rapid affinity/kinetic screens. Most large libraries made to date use the single-chain format for display on phage (7, 19). One report described the use of a human non-immunized Fab library on phage (not permitting immediate screening of selected soluble Fab fragments) (35). scFv fragments have the tendency to form dimers and higher order multimers in a clone-dependent and relatively unpredictable way (20–22). As a consequence, the affinity assay used (such as BIAcore analysis) often necessitates purification of the selected antibody fragments. For example, ranking for off-rates using BIAcore is not easily possible with unpurified scFv fragments; the monomeric fraction of selected scFv clones first needs to be purified by affinity chromatography and gel filtration (19, 24). Our data suggest that the off-rate screening of individual Fab clones using non-purified bacterial preparations yield data similar to the off-rates determined with the purified Fab fragments. Therefore, provided sufficient Fab fragment is produced, the true monomeric appearance of Fabs allows a rapid initial screen for off-rate. In combination with a concentration

TABLE IV
V-gene segments and CDR3 sequences used by the selected Fabs

Clone	Specificity	VH family	VH segment	CDR3	Amino acid changes from germline ^a	VL family	VL segment	CDR3	Amino acid changes from germline ^a
MUC#4	MUC1	VH4	4-39	AAAGMVD	7	V κ 2	A17	MQATHWPPIT	1
MUC#9	MUC1	VH4	4-b/4-39	PSIAAAGQVY	5	V κ 3	L6	QQYYSWPLT	10
MUC#32	MUC1	VH3	3-30	VGSGGWLYFDR	2	V κ 3	L6	QQRSHWPLT	2
CG#4F	β -CG	VH3	3-30.3	EGTATPGGTDY	2	V κ 1	L5	QQSYSTPL	7
CG#5C	β -CG/LH	VH4	4-04	GAAASYFYDY	0	V λ 6	6a	QSSHSTAVV	13
LH#1C	α	VH1	1-08	GERSNFDY	8	V κ 3	L6	QHRRT	8
LH#2H	$\alpha\beta$ -LH	VH3	3-15	DPGTIIIIYYGMDV	0	V κ 2	A19	MQALQAPLT	4
LH#3F	α	VH3	3-30.5	LYGDYVSADF	2	V λ 7	7b	LLYYGGARGV	6
LH#3G	$\alpha\beta$ -LH	VH3	3-15	RIAAYYYYGMDV	0	V κ 2	A19	MQALQTPRT	0
FS#8B	β -FSH	VH6	6-01	GEHGYTSS	8	V κ 2	A19	MQALQTPPT	1
SC#2B	α	VH1	1-02	GKVGASFDY	3	V λ 2	2a2	SSYTNSGTLV	9
SC#2F	α	VH1	1-02	ASGYFPNDAFDI	6	V λ 1	1c	ESLDDSLDGVV	20
SC#2G	α	VH1	1-e	GEASYGAYNWFD	5	V λ 2	2e	CSYAGSNTWV	9
SC#4G	α	VH1	1-24	GGYSGLA	2	V κ 2	A19	MQTLQPPWT	2

^a Amino acid differences in V-gene segment, excluding the FR-1 region encoded by the primers used for cloning, and CDR3.

TABLE V
Affinities of anti-hormone Fabs for hCG

Clone	Preparation	k_{on} s^{-1}	k_{off} $M^{-1}s^{-1}$	K_d M
LH#1C	Purified	$(1.04 \pm 0.04) \times 10^{-2}$	$(4.50 \pm 0.37) \times 10^5$	$(2.30 \pm 0.23) \times 10^{-8}$
	Periplasmic fraction	$(1.12 \pm 0.08) \times 10^{-2}$	ND ^a	
SC#2B	Purified	$(2.89 \pm 0.41) \times 10^{-3}$	$(1.06 \pm 0.08) \times 10^6$	$(2.71 \pm 0.25) \times 10^{-9}$
	Periplasmic fraction	$(2.90 \pm 0.57) \times 10^{-3}$	ND	
CG#5C	Purified	$(1.46 \pm 0.27) \times 10^{-2}$	$(3.76 \pm 0.71) \times 10^5$	$(3.88 \pm 0.03) \times 10^{-8}$
	Periplasmic fraction	$(1.21 \pm 0.16) \times 10^{-2}$	ND	
LH#3F	Periplasmic fraction	$(5.84 \pm 0.08) \times 10^{-3}$	ND	ND
CG#4F	Periplasmic fraction	$(2.22 \pm 0.28) \times 10^{-3}$	ND	ND
SC#2F	Periplasmic fraction	2.25×10^{-3}	ND	ND

^a ND, not determined.

determination assay (which could also be carried out on BIAcore; Ref. 34), this should allow the rapid affinity determination of large series of antigen-specific Fabs. The Fab format is therefore more amenable than scFv to high throughput affinity screening, and should be the preferred format when rapid affinity measurement is crucial (e.g. during affinity maturation studies).

Most large libraries made to date use the single-chain format for display on phage (7, 19), which does not easily allow the rapid screening of large numbers of clones on kinetics of binding (off-rate) with crude protein fractions. One report described a very large human synthetic library with Fab fragments displayed on phage, which was constructed with an *in vivo* recombination system to combine separately cloned heavy (with completely synthetic CDR3 sequences) and light chain repertoires (with few randomized CDR3 residues) (35). Although the authors also used affinity-purified Fab fragments for affinity measurements without further purification by gel filtration, screening of individual clones had to be performed after recloning of the selected Fabs for soluble expression. Clearly, this system does not allow a rapid screening procedure, while the low percentage (28%) of clones having both a heavy and a light chain after the recombination event suggests instability of the library.

As was postulated and observed by Griffiths and colleagues (35), the size of the antibody library dictates the probability of the selection of high affinity antibodies to the antigen. Comparison of the first non-immunized scFv repertoire containing 2.9×10^7 clones (4), with recently constructed scFv repertoires of approximately 10^{10} clones (7, 19), confirms this postulation; increasing the library size 500-fold resulted in approximately 100-fold higher affinities. This increase is caused by lowering the off-rates from 10^{-1} to $10^{-2} s^{-1}$ for fragments selected from the smaller sized library to 10^{-3} to $10^{-4} s^{-1}$ for those from the

larger library. This is in the same order of magnitude as we observe for the off-rates of our selected antibody fragments. Since Fab fragments lack the tendency to dimerize, Fab libraries could possibly display a lower fraction of avid phage than equivalent scFv libraries library. This does not appear to have reduced the number or average affinity of selected antibodies. An indication that antibodies from this library behave similarly or better with regards to affinity comes from a comparison of selections of two different libraries on the same two antigens under identical conditions. Antibodies to MUC1 selected from a large non-immunized scFv library (29) have faster off-rates than the equivalent Fabs isolated from the library described in this study. Further, they show a very distinct V-gene usage and have a different fine specificity.⁴ Similarly, when comparing the off-rates of phage antibodies against the pancreatic carcinoma marker epithelial glycoprotein-2, one of the Fabs selected from the present library appears to have a 10-fold slower off-rate than the best scFv (7).⁵

The affinities of the selected antibody fragments are, however, very dependent on the antigen used for selection. Sheets and colleagues reported an affinity varying between 26 and 71 nM for the selected scFv fragments specific for the anti-*Clostridia botulinum* neurotoxin type A fragments, whereas for antibodies to the extracellular domain of human ErbB-2, K_d values between 0.22 and 4.03 nM were found (19). The affinities of the gonadotropin-specific Fabs selected from our library varied between 2.7 and 38 nM, which is comparable to the protein binding scFv fragments from the non-immunized library made

⁴ P. Henderikx, K. E. Tengberg, R. Hoet, C. Petrarca, E. van der Linden, A. de Bruijn, J. Zeuthen, J. W. Arends, and H. R. Hoogenboom, manuscript in preparation.

⁵ R. C. Roovers, E. van der Linden, A. de Bruijn, J. W. Arends, D. C. Boerman, and H. R. Hoogenboom, manuscript in preparation.

by Vaughan *et al.* (7) and Sheets *et al.* (19). It also approaches the values of the best antibodies in their kind.⁶

The size of the library is not only important for affinity, it also determines the success rate of selection of antibodies against a large set of different antigens. In this respect the library performs very well; over 24 antibodies to the hapten phOx, and on average 13 antibodies against the other antigens were selected. Furthermore, the specificities of the antibodies obtained by selections on the gonadotropins are unique; due to the high degree of homology between hLH and hCG, it has been very difficult to isolate hCG-specific monoclonal antibodies with the hybridoma technology, whereas there are very few hLH-specific antibodies (32, 42). Using a straightforward selection procedure, taking no precaution to avoid the selection of cross-reactive Fabs, we have readily isolated fragments with all possible specificities: Fabs specific for any of the three hormones hCG, hLH, and hFSH, and cross-reactive Fabs recognizing the common α -chain or epitopes on the β -chain shared by hCG and hLH. These selections demonstrated that antibodies directed against different epitopes within single antigen molecules can be retrieved from the library.

In the limited set of 14 clones that were sequenced, we identified antibodies with variable region genes from all large V-gene families, including VH1/3/4, V κ 1/3, and V λ 1/2; but less frequently used segments of family VH6, V κ 2/7, and V λ 7 were also retrieved. Most likely, the use of an extended set of variable region gene primers, designed on the most recent sequence information of the germline V-regions, and/or the separate PCRs, combined with partially separate cloning, ensured access to a highly diverse sample of the human V-gene repertoire. The average amino acid mutation frequency of the selected human V-genes was calculated to be 4.0% for the VH segments (50 amino acid mutations in 1267) and 7.3% for the VL (92 out of 1260). This mutation frequency is the reverse of that reported for IgG+ B cells (52) (10% for VH and 6% for VL). The higher mutation rate of the VL-genes in the combinatorial repertoire may serve to contribute the rather restricted natural light chain diversity. With respect to the VH segments of the selected antibodies, the mutation frequency is remarkably lower (mean 3.57 (\pm 2.90) mutated residues per VH) than what was found in the selected scFv fragments by Vaughan and colleagues (7) (mean 7.53 (\pm 4.25) mutated residues). This is most likely caused by the amplifications with VH-based primers for the construction of the latter library, instead of the IgM primer used by us for the primary PCRs. The light chain segments seem to have a similar mutation frequency (Vaughan *et al.* reported 8.23 \pm 5.20 residues, versus 6.57 \pm 5.56 residues in the Fabs reported here).

This new phage library will be a valuable source of antibodies to essentially any target. To date, we have been able to select specific antibodies to over 20 antigens tested. The antibodies may be used as research reagents or as a starting point for the development of therapeutic antibodies. As the list of sequenced genomes and disease-related gene products is expanding rapidly, there will be a growing need for an *in vitro* and eventually automated method for antibody isolation. As antibodies have been and will be ideal probes for investigating the nature, localization, and purification of novel gene products, this library is envisaged to play an important role in target validation and target discovery in the area of functional genomics.

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